FORM PTO-1390 (Rev 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **PATKRI P01AUS**

U.S. APPLICATION NO (If known, see 37 C.F.R 1.5): **09/856543**

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP99/08888	**November 19, 1999**	**November 23, 1998**

TITLE OF INVENTION

METHOD FOR THE ELECTROCHEMICAL DETECTION OF NUCLEIC ACID OLIGOMER HYBRIDS

APPLICANT(S) FOR DO/EO/US

Gerhard HARTWICH and Adam HELLER

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ■ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ■ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ■ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ■ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ■ has been transmitted by the International Bureau. (PCT/IB/308 mailed **02 June 2000**).
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ■ A translation of the International Application into English (35 U.S.C. 371(c)(2)) is attached.
7. ■ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ■ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S C. 371(c)(3)).
9. ■ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (unsigned)
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ■ An Information Disclosure Statement under 37 CFR 1.97 and 1.98 with PTO FORM 1449.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ■ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ■ Other items or information:

■ Preliminary Examination Report	■ Copy of Request
■ Annexes to Pre. Ex. Rep.	■ 4 sheets of formal drawings
■ International Search Report	■ Abstract
☐ German Novelty Search Report	■ German Language Specification
■ 51 copies of citations	☐ Copy of Notification of File Missing Parts
■ Form PCT/IB/308	☐
■ International Publ. No. **WO 00/31101** (Face page only)	

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this Transmittal Letter and the papers indicated as being transmitted therewith is being deposited with the United States Postal Service on this date **May 22, 2001** in an envelope as "Express Mail Post Office to Addressee" Mailing Label No. **EL469354882US** addressed to the: Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Michael J. Bujold
(typed or printed name of person mailing paper)

(signature of person mailing paper)

17. ■ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO $860.00	JC18 Rec'd PCT/PTO 22 MAY 200	
International preliminary examination fee paid to USPTO (37 CFR 1.482) $690.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)). $710.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $1000.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	860	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	0	

Claims	Number Filed	Number Extra	Rate		
Total Claims	55-20 =	35	x $18.00	630	
Independent Claims	4-3 =	1	x $80.00	80	
Multiple dependent claim(s) (if applicable)			+ $270.00	0	
			TOTAL OF ABOVE CALCULATIONS =	1570	
Reduction by 1/2 for filing by small entity, if applicable. **Applicant Claims Small Entity Status.** (Note 37 CFR 1.9, 1.27, 1.28).				785	
			SUBTOTAL =	785	
Processing fee of $130.00 for furnishing the English translation later the ☐ 20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)).			+	0	
			TOTAL NATIONAL FEE =	0	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property			+	0	
			TOTAL FEES ENCLOSED =	785	
			Amount to be: refunded		$
			charged		$

a. ■ A check in the amount of $ **785.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 04-0213 in the amount of $____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ■ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-0213 . A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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PATENT & TRADEMARK OFFICE

020210

08/24/01 PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	:	Gerhard HARTWICH and Adam HELLER
Serial no.	:	09/856,543
Filed	:	an effective filing date of November 19, 1999
For	:	METHOD FOR THE ELECTROCHEMICAL DETECTION OF NUCLEIC ACID OLIGOMER HYBRIDS
Group Art Unit	:	
Examiner	:	
Docket	:	PATKRI P01AUS

The Commissioner of Patents and Trademarks
Washington, D.C. 20231

RESPONSE TO NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 AND AMENDMENT TO INCORPORATE SEQUENCE LISTING

Dear Sir:

[XXX] NO FEES ARE PAYABLE WITH RESPECT TO THIS RESPONSE.

On June 26, 2001, a NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. § 371 (referred to herein as NOTIFICATION) was mailed from the Patent Office. This paper is filed in partial response to the Notification. Please amend the application as described below.

In the Specification:

Please cancel te following: the Table on pages 4-9; the table on page 21; page 24, first full paragraph; page 24, last full paragraph through to page 25 top paragraph; page 25, second full paragraph of the specification, in their entirety, in favor of a clean form of the Table on pages 4-9; the table on page 21; page 24, first full paragraph; page 24, last full paragraph through to page 25 top paragraph; page 25, second full paragraph of the specification, without any markings thereon, as follows. Also accompanying this response is a copy of the original paragraphs of the specification which show the addition(s) (by underlining, shading and bold) and the deletion(s) (by strikeout) to the canceled specification paragraphs. Please enter the replacement specification paragraphs into the record of this case.

Please insert the attached Sequence Listing after page 26 of the specification, and renumber the claims pages to begin with 28.

TABLE ON PAGES 4-9

Genetics	
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
PNA	peptide nucleic acid (Synthetic DNA or RNA in which the sugar-phosphate moiety is replaced by an amino acid. If the sugar-phosphate moiety is replaced by the -NH-$(CH_2)_2$-N($COCH_2$-base)-CH_2CO- moiety, PNA will hybridize with DNA.)
A	adenine
G	guanine
C	cytosine
T	thymine
base	A, G, T, or C
bp	base pair
nucleic acid	At least two covalently joined nucleotides or at least two covalently joined pyrimidine (e.g. cytosine, thymine, or uracil) or purine bases (e.g. adenine or guanine). The term nucleic acid refers to any backbone of the covalently joined pyrimidine or purine bases, such as e.g. to the sugar-phosphate backbone of DNA, cDNA, or RNA, to a peptide backbone of PNA, or to analogous structures (e.g. a phosphoramide, thiophosphate, or dithiophosphate backbone). The essential feature of a nucleic acid according to the present invention is that it can sequence-specifically bind naturally occurring cDNA or RNA.
nucleic acid oligomer	Nucleic acid of base length that is not further specified (e.g. nucleic acid octamer: a nucleic acid having any backbone in which 8 pyrimidine or purin bases are covalently bound to one another).
oligomer	Equivalent to nucleic acid oligomer.
oligonucleotide	Equivalent to oligomer or nucleic acid oligomer, thus e.g. a DNA, PNA, or RNA fragment of base length that is not further

	specified.
oligo	Abbreviation for oligonucleotide.
dATP	Deoxyribonucleoside triphosphate of A (DNA moiety with the A base and two further phosphates to build a longer DNA fragment or oligonucleotide).
dGTP	Deoxyribonucleoside triphosphate of G (DNA moiety with the G base and two further phosphates to build a longer DNA fragment or oligonucleotide).
dCTP	Deoxyribonucleoside triphosphate of C (DNA moiety with the C base and two further phosphates to build a longer DNA fragment or oligonucleotide).
dTTP	Deoxyribonucleoside triphosphate of T (DNA moiety with the T base and two further phosphates to build a longer DNA fragment or oligonucleotide).
primer	Initial complementary fragment of an oligonucleotide, with the base length of the primer being only approx. 4-8 bases. Serves as the starting point for enzymatic replication of an oligonucleotide.
mismatch	To form the Watson Crick double-stranded oligonucleotide structure, the two single strands hybridize in such a way that the A (or C) base of one strand forms hydrogen bonds with the T (or G) base of the other strand (in RNA, T is replaced by uracil). Any other base pairing does not form hydrogen bonds, distorts the structure, and is referred to as a "mismatch."
ds	double strand
ss	single strand
Chemical Substances/Groups	
R	A substituent or side chain of any organic residue not further specified.
redox	redox-active substance
alkyl	The term "alkyl" refers to a saturated hydrocarbon radical that is straight-chained or branched (e.g. ethyl, isopropyl, or 2,5-

	dimethylhexyl, etc.). When "alkyl" is used to indicate a linker or spacer, the term refers to a group having two available valences for covalent linkage (e.g. $-CH_2CH_2-$, $-CH_2CH_2CH_2-$, or $-CH_2C(CH_3)_2CH_2CH_2C(CH_3)_2CH_2-$, etc.). Alkyl groups preferred as substituents or side chains R are those of chain length 1-30 (longest continuous chain of atoms bound to one another). Alkyl groups preferred as linkers or spacers are those of chain length 1-20, especially of chain length of 1-14, the chain length representing the shortest continuous link between linker or spacer-joined structures.
alkenyl	Alkyl groups in which one or more of the C-C single bonds are replaced by C=C double bonds.
alkinyl	Alkyl or alkenyl groups in which one or more of the C-C single or C=C double bonds are replaced by C≡C triple bonds.
heteroalkyl	Alkyl groups in which one or more of the C-H bonds or C-C single bonds are replaced by C-N, C=N, C-P, C=P, C-O, C=O, C-S, or C=S bonds.
heteroalkenyl	Alkenyl groups in which one or more C-H bonds, C-C single, or C=C double bonds are replaced by C-N, C=N, C-P, C=P, C-O, C=O, C-S, or C=S bonds.
heteroalkinyl	Alkinyl groups in which one or more of the C-H bonds, C-C single, C=C double, or C≡C triple bonds are replaced by C-N, C=N, C-P, C=P, C-O, C=O, C-S, or C=S bonds.
linker	A molecular link between two molecules or between a surface atom, surface molecule, or surface molecule group and another molecule. Linkers can usually be purchased in the form of alkyl, alkenyl, alkinyl, heteroalkyl, heteroalkenyl, or heteroalkinyl chains, the chain being derivatized in two places with (identical or different) reactive groups. These groups form a covalent chemical bond in simple/known chemical reactions with the appropriate reaction partner. The reactive groups may also be photoactivatable, i.e. the reactive groups are activated only

	by light of a specific or random wavelength. Preferred linkers are those of chain length of 1-20, especially of chain length of 1-14, the chain length representing here the shortest continuous link between the structures to be joined, thus between the two molecules or between a surface atom, surface molecule, or surface molecule group and another molecule.
spacer	A linker that is covalently attached via the reactive groups to one or both of the structures to be joined (see linker). Preferred spacers are those of chain length 1-20, especially of chain length 1-14, the chain length representing the shortest continuous link between the structures to be joined.
(n x HS-spacer)-oligo	A nucleic acid oligomer to which n thiol functions are each attached via a spacer, where each spacer may have a different chain length (shortest continuous link between the thiol function and the nucleic acid oligomer), especially any chain length between 1 and 14 each. These spacers, in turn, may be bound to various reactive groups that are naturally present on the nucleic acid oligomer or that have been fixed thereto by means of modification, and "n" is any integer, especially a number between 1 and 20.
(n x R-S-S-spacer)-oligo	A nucleic acid oligomer to which n disulfide functions are each attached via a spacer, and any residue R saturates the disulfide function. Each spacer for attaching the disulfide function to the nucleic acid oligomer may have a different chain length (shortest continuous link between the disulfide function and the nucleic acid oligomer), especially any chain length between 1 and 14 each. These spacers, in turn, may be bound to various reactive groups that are naturally present on the nucleic acid oligomer or that have been fixed thereto by means of modification. The placeholder "n" is any integer, especially a number between 1 and 20.
oligo-spacer-S-S-	Two identical or different nucleic acid oligomers that are joined to

spacer-oligo	each other via a disulfide bridge, the disulfide bridge being attached to the nucleic acid oligomers via any two spacers and the two spacers potentially having differing chain lengths (shortest continuous link between the disulfide bridge and the respective nucleic acid oligomer), especially any chain length between 1 and 14 each, and these spacers, in turn, potentially being bound to various reactive groups that are naturally present on the nucleic acid oligomer or that have been fixed thereto by means of modification.
PQQ	pyrroloquinoline quinone; corresponds to 4,5-dihydro-4,5-dioxo-1H-pyrrolo-[2,3-f]-quinoline-2,7,9-tricarboxylic acid)
TEATFB	tetraethylammonium-tetrafluoroborate
sulfo-NHS	N-hydroxysulfosuccinimide
EDC	(3-dimethylaminopropyl)-carbodiimide
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
Tris	trishydroxymethylamino methane
EDTA	ethylenediamine tetraacetate (sodium salt)
cystamine	$(H_2N\text{-}CH_2\text{-}CH_2\text{-}S\text{-})_2$
Modified Surfaces/Electrodes	
mica	Muskovite platelets, a support for the application of thin layers.
Au-S-ss-oligo-PQQ	Gold film on mica having a covalently applied monolayer of derivatized 12-bp single-strand oligonucleotide (sequence: TAGTCGGAAGCA) SEQ ID NO: 1. Here, the terminal phosphate group of the oligonucleotide at the 3' end is esterified with $(HO\text{-}(CH_2)_2\text{-}S)_2$ to $P\text{-}O\text{-}(CH_2)_2\text{-}S\text{-}S\text{-}(CH_2)_2\text{-}OH$, homolytically cleaving the S-S bond and producing one Au-S-R bond each. The terminal thymine base at the 5' end of the oligonucleotide is modified at the C-5 carbon with $\text{-}CH{=}CH\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}NH_2$ and the residue, in turn, is joined via its free amino group with a carboxylic-acid group of the PQQ by means of amidation.

Au-S-ds-oligo-PQQ	Au-S-ss-oligo-PQQ that is hybridized with the oligonucleotide complementary to the ss-oligo (sequence: TAGTCGGAAGCA SEQ ID NO: 1).
Electrochemistry	
E	The electrode potential on the working electrode.
E_0	Half-wave potential, the potential in the middle between the current maximums for oxidation and reduction of cyclic voltammetrically reversible electrooxidation or reduction.
i	current density (current per cm^2 of electrode surface)
cyclic voltammetry	Recording a current-voltage curve. The potential of a stationary working electrode is changed linearly as a function of time, starting at a potential at which no electrooxidation or reduction occurs, up to a potential at which a species that is solute or adsorbed on the electrode is oxidized or reduced (i.e. current flows). After running through the oxidation or reduction operation, which produces in the current-voltage curve an initially increasing current and, after reaching a maximum, a gradually decreasing current, the direction of the potential feed is reversed. The behavior of the products of electrooxidation or electroreduction is then recorded in reverse run.
amperometry	Recording a current-time curve. Here, the potential of a stationary working electrode is set, e.g. by means of a potential jump, to a potential at which the electrooxidation or reduction of a solute or adsorbed species occurs, and the flowing current is recorded as a function of time.

TABLE ON PAGE 21

Fig. 1	Shows a schematic illustration of the Sanger method of oligonucleotide sequencing;
Fig. 2	Shows a schematic illustration of oligonucleotide sequencing by means of hybridization on a chip;
Fig. 3	Shows a schematic illustration of the surface hybrid of the general structure elec-spacer-ss-oligo-spacer-redox with a 12-bp probe oligonucleotide of the exemplary sequence 5'-TAGTCGGAAGCA-3' SEQ ID NO: 1 (left) and Au-S-ss-oligo-PQQ in the hybridized state as an embodiment example of an elec-spacer-ss-oligo-spacer-redox; only a portion of the probe oligonucleotide having a hybridized complementary strand is shown (right), the attachment of the oligonucleotide to the surface redox-active substance PQQ occurred via the spacer $-CH_2-CH=CH-CO-NH-CH_2-CH_2-NH-$;
Fig. 4	Shows a cyclic voltammogram of a test site consisting of Au-S-ss-oligo-PQQ (dotted) compared with an identical test site with completely hybridized target (Au-S-ds-oligo-PQQ, solid line);
Fig. 5	Shows a cyclic voltammogram of a test site with completely hybridized target (Au-S-ds-oligo-PQQ) (solid line) compared with a test site with hybridized target that exhibits 2 base-pair mismatches (Au-S-ds-oligo-PQQ with 2 bp mismatches, broken)

PAGE 24, FIRST FULL PARAGRAPH

To prepare the ds oligonucleotide solution, a double-modified 12-bp single-strand oligonucleotide of the sequence 5'-TAGTCGGAAGCA-3' SEQ ID NO: 1 was used, which is esterified with $(HO\text{-}(CH_2)_2\text{-}S)_2$ at the phosphate group of the 3' end to P-O-$(CH_2)_2$-S-S-$(CH_2)_2$-OH. At the 5' end, the terminal base of the oligonucleotide, thymine, is modified at the C-5 carbon with -CH=CH-CO-NH-CH_2-CH_2-NH_2. A $2x10^{-4}$ molar solution of this oligonucleotide in the hybridization buffer (10 mM Tris, 1 mM EDTA, pH 7.5 with 0.7 molar addition of TEATFB, see abbreviations) was hybridized with a $2x10^{-4}$ molar solution of the (unmodified) complementary strand in the hybridization buffer at room temperature for approx. 2 hours (hybridization step). During a reaction time of approx. 12-24 h, the disulfide spacer P-O-$(CH_2)_2$-S-S-$(CH_2)_2$-OH of the oligonucleotide was homolytically cleaved. In this process, the spacer forms a covalent Au-S bond with the Au atoms of the surface, thus causing to a 1:1 coadsorption of the ds-oligonucleotide and the 2-hydroxy-mercaptoethanol.

PAGE 24, LAST FULL PARAGRAPH-PAGE 25 TOP PARAGRAPH

Example 2: Producing the Au-S-ss-oligo-PQQ oligonucleotide electrode. Analogously to the production of the Au-S-ds-oligo-PQQ system, the support surface is derivatized with modified single-strand oligonucleotide, dispensing with only the hybridization of the modified oligonucleotide of the sequence 5'-TAGTCGGAAGCA-3' SEQ ID NO: 1 with its complementary strand and, in the incubation step, using only the double-modified 12-bp single-strand probe oligonucleotide (see Example 1) in the form of a 1×10^{-4} molar solution in water and in the presence of 10^{-2} molar Tris, 10^{-3} molar EDTA and 0.7 molar TEATFB (or 1 molar NaCl) at pH 7.5. The redox step was carried out as indicated in Example 1.

PAGE 25, SECOND FULL PARAGRAPH

Example 3: Producing the Au-S-ds-oligo-PQQ oligonucleotide electrode having 2 bp mismatches. The production of a support surface derivatized with modified double-strand oligonucleotide was carried out analogously to the production of the Au-S-ds-oligo-PQQ system, but only in hybridizing the modified oligonucleotide of the sequence 5'-TAGTCGGAAGCA-3' SEQ ID NO: 1 was a complementary strand used (sequence: 5'-ATCAG***AT***TTCGT-3') SEQ ID NO: 2, in which bases no. 6 and 7 (counted from the 5' end), which are actually complementary, were modified from C to ***A*** or from C to ***T*** to introduce two base-pair mismatches.

REMARKS

In response to the Notification, attached is a Sequence Listing. Also transmitted herewith is a copy of the Sequence Listing in computer readable form. As required by 37 CFR 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d) Applicants' Attorney hereby states that the content of the Sequence Listing in paper form and on the computer readable form of the Sequence Listing are the same, and the submission includes no new matter.

A Declaration and Power of Attorney, signed by the inventor will follow in due course.

In the event that there are any fee deficiencies or additional fees are payable, please charge the same or credit any overpayment to our Deposit Account (Account No. 04-0213).

Respectfully submitted,

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CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service, with sufficient postage, as First Class Mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231 on August 24, 2001 .

By: _____

Print Name: Michael J. Bujold

VERSION WITH MARKINGS TO SHOW CHANGES MADE

TABLE ON PAGES 4-9

Genetics	
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
PNA	peptide nucleic acid (Synthetic DNA or RNA in which the sugar-phosphate moiety is replaced by an amino acid. If the sugar-phosphate moiety is replaced by the -NH-$(CH_2)_2$-N($COCH_2$-base)-CH_2CO- moiety, PNA will hybridize with DNA.)
A	adenine
G	guanine
C	cytosine
T	thymine
base	A, G, T, or C
bp	base pair
nucleic acid	At least two covalently joined nucleotides or at least two covalently joined pyrimidine (e.g. cytosine, thymine, or uracil) or purine bases (e.g. adenine or guanine). The term nucleic acid refers to any backbone of the covalently joined pyrimidine or purine bases, such as e.g. to the sugar-phosphate backbone of DNA, cDNA, or RNA, to a peptide backbone of PNA, or to analogous structures (e.g. a phosphoramide, thiophosphate, or dithiophosphate backbone). The essential feature of a nucleic acid according to the present invention is that it can sequence-specifically bind naturally occurring cDNA or RNA.
nucleic acid oligomer	Nucleic acid of base length that is not further specified (e.g. nucleic acid octamer: a nucleic acid having any backbone in which 8 pyrimidine or purin bases are covalently bound to one another).
oligomer	Equivalent to nucleic acid oligomer.
oligonucleotide	Equivalent to oligomer or nucleic acid oligomer, thus e.g. a DNA, PNA, or RNA fragment of base length that is not further specified.
oligo	Abbreviation for oligonucleotide.
dATP	Deoxyribonucleoside triphosphate of A (DNA moiety with the A base and two further phosphates to build a longer DNA fragment or oligonucleotide).

dGTP	Deoxyribonucleoside triphosphate of G (DNA moiety with the G base and two further phosphates to build a longer DNA fragment or oligonucleotide).
dCTP	Deoxyribonucleoside triphosphate of C (DNA moiety with the C base and two further phosphates to build a longer DNA fragment or oligonucleotide).
dTTP	Deoxyribonucleoside triphosphate of T (DNA moiety with the T base and two further phosphates to build a longer DNA fragment or oligonucleotide).
primer	Initial complementary fragment of an oligonucleotide, with the base length of the primer being only approx. 4-8 bases. Serves as the starting point for enzymatic replication of an oligonucleotide.
mismatch	To form the Watson Crick double-stranded oligonucleotide structure, the two single strands hybridize in such a way that the A (or C) base of one strand forms hydrogen bonds with the T (or G) base of the other strand (in RNA, T is replaced by uracil). Any other base pairing does not form hydrogen bonds, distorts the structure, and is referred to as a "mismatch."
ds	double strand
ss	single strand
Chemical Substances/Groups	
R	A substituent or side chain of any organic residue not further specified.
redox	redox-active substance
alkyl	The term "alkyl" refers to a saturated hydrocarbon radical that is straight-chained or branched (e.g. ethyl, isopropyl, or 2,5-dimethylhexyl, etc.). When "alkyl" is used to indicate a linker or spacer, the term refers to a group having two available valences for covalent linkage (e.g. $-CH_2CH_2-$, $-CH_2CH_2CH_2-$, or $-CH_2C(CH_3)_2CH_2CH_2C(CH_3)_2CH_2-$, etc.). Alkyl groups preferred as substituents or side chains R are those of chain length 1-30 (longest continuous chain of atoms bound to one another). Alkyl groups preferred as linkers or spacers are those of chain length 1-20, especially of chain length of 1-14, the chain length representing

	the shortest continuous link between linker or spacer-joined structures.
alkenyl	Alkyl groups in which one or more of the C-C single bonds are replaced by C=C double bonds.
alkinyl	Alkyl or alkenyl groups in which one or more of the C-C single or C=C double bonds are replaced by C≡C triple bonds.
heteroalkyl	Alkyl groups in which one or more of the C-H bonds or C-C single bonds are replaced by C-N, C=N, C-P, C=P, C-O, C=O, C-S, or C=S bonds.
heteroalkenyl	Alkenyl groups in which one or more C-H bonds, C-C single, or C=C double bonds are replaced by C-N, C=N, C-P, C=P, C-O, C=O, C-S, or C=S bonds.
heteroalkinyl	Alkinyl groups in which one or more of the C-H bonds, C-C single, C=C double, or C≡C triple bonds are replaced by C-N, C=N, C-P, C=P, C-O, C=O, C-S, or C=S bonds.
linker	A molecular link between two molecules or between a surface atom, surface molecule, or surface molecule group and another molecule. Linkers can usually be purchased in the form of alkyl, alkenyl, alkinyl, heteroalkyl, heteroalkenyl, or heteroalkinyl chains, the chain being derivatized in two places with (identical or different) reactive groups. These groups form a covalent chemical bond in simple/known chemical reactions with the appropriate reaction partner. The reactive groups may also be photoactivatable, i.e. the reactive groups are activated only by light of a specific or random wavelength. Preferred linkers are those of chain length of 1-20, especially of chain length of 1-14, the chain length representing here the shortest continuous link between the structures to be joined, thus between the two molecules or between a surface atom, surface molecule, or surface molecule group and another molecule.
spacer	A linker that is covalently attached via the reactive groups to one or both of the structures to be joined (see linker). Preferred spacers are those of chain length 1-20, especially of chain length 1-14, the chain length representing the shortest continuous link between the structures to be joined.

(n x HS-spacer)-oligo	A nucleic acid oligomer to which n thiol functions are each attached via a spacer, where each spacer may have a different chain length (shortest continuous link between the thiol function and the nucleic acid oligomer), especially any chain length between 1 and 14 each. These spacers, in turn, may be bound to various reactive groups that are naturally present on the nucleic acid oligomer or that have been fixed thereto by means of modification, and "n" is any integer, especially a number between 1 and 20.
(n x R-S-S-spacer)-oligo	A nucleic acid oligomer to which n disulfide functions are each attached via a spacer, and any residue R saturates the disulfide function. Each spacer for attaching the disulfide function to the nucleic acid oligomer may have a different chain length (shortest continuous link between the disulfide function and the nucleic acid oligomer), especially any chain length between 1 and 14 each. These spacers, in turn, may be bound to various reactive groups that are naturally present on the nucleic acid oligomer or that have been fixed thereto by means of modification. The placeholder "n" is any integer, especially a number between 1 and 20.
oligo-spacer-S-S-spacer-oligo	Two identical or different nucleic acid oligomers that are joined to each other via a disulfide bridge, the disulfide bridge being attached to the nucleic acid oligomers via any two spacers and the two spacers potentially having differing chain lengths (shortest continuous link between the disulfide bridge and the respective nucleic acid oligomer), especially any chain length between 1 and 14 each, and these spacers, in turn, potentially being bound to various reactive groups that are naturally present on the nucleic acid oligomer or that have been fixed thereto by means of modification.
PQQ	pyrroloquinoline quinone; corresponds to 4,5-dihydro-4,5-dioxo-1H-pyrrolo-[2,3-f]-quinoline-2,7,9-tricarboxylic acid)
TEATFB	tetraethylammonium-tetrafluoroborate
sulfo-NHS	N-hydroxysulfosuccinimide
EDC	(3-dimethylaminopropyl)-carbodiimide

HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
Tris	trishydroxymethylamino methane
EDTA	ethylenediamine tetraacetate (sodium salt)
cystamine	$(H_2N\text{-}CH_2\text{-}CH_2\text{-}S\text{-})_2$
Modified Surfaces/Electrodes	
mica	Muskovite platelets, a support for the application of thin layers.
Au-S-ss-oligo-PQQ	Gold film on mica having a covalently applied monolayer of derivatized 12-bp single-strand oligonucleotide (sequence: TAGTCGGAAGCA) SEQ ID NO: 1. Here, the terminal phosphate group of the oligonucleotide at the 3' end is esterified with $(HO\text{-}(CH_2)_2\text{-}S)_2$ to $P\text{-}O\text{-}(CH_2)_2\text{-}S\text{-}S\text{-}(CH_2)_2\text{-}OH$, homolytically cleaving the S-S bond and producing one Au-S-R bond each. The terminal thymine base at the 5' end of the oligonucleotide is modified at the C-5 carbon with $\text{-}CH{=}CH\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}NH_2$ and the residue, in turn, is joined via its free amino group with a carboxylic-acid group of the PQQ by means of amidation.
Au-S-ds-oligo-PQQ	Au-S-ss-oligo-PQQ that is hybridized with the oligonucleotide complementary to the ss-oligo (sequence: TAGTCGGAAGCA SEQ ID NO: 1).
Electrochemistry	
E	The electrode potential on the working electrode.
E_0	Half-wave potential, the potential in the middle between the current maximums for oxidation and reduction of cyclic voltammetrically reversible electrooxidation or reduction.
i	current density (current per cm^2 of electrode surface)
cyclic voltammetry	Recording a current-voltage curve. The potential of a stationary working electrode is changed linearly as a function of time, starting at a potential at which no electrooxidation or reduction occurs, up to a potential at which a species that is solute or adsorbed on the electrode is oxidized or reduced (i.e. current flows). After running through the oxidation or reduction operation, which produces in the current-voltage curve an initially increasing current and, after reaching a maximum, a gradually decreasing current, the direction of the potential feed is reversed. The behavior of the products of

	electrooxidation or electroreduction is then recorded in reverse run.
amperometry	Recording a current-time curve. Here, the potential of a stationary working electrode is set, e.g. by means of a potential jump, to a potential at which the electrooxidation or reduction of a solute or adsorbed species occurs, and the flowing current is recorded as a function of time.

TABLE ON PAGE 21

Fig. 1	Shows a schematic illustration of the Sanger method of oligonucleotide sequencing;
Fig. 2	Shows a schematic illustration of oligonucleotide sequencing by means of hybridization on a chip;
Fig. 3	Shows a schematic illustration of the surface hybrid of the general structure elec-spacer-ss-oligo-spacer-redox with a 12-bp probe oligonucleotide of the exemplary sequence 5'-TAGTCGGAAGCA-3' SEQ ID NO: 1 (left) and Au-S-ss-oligo-PQQ in the hybridized state as an embodiment example of an elec-spacer-ss-oligo-spacer-redox; only a portion of the probe oligonucleotide having a hybridized complementary strand is shown (right), the attachment of the oligonucleotide to the surface redox-active substance PQQ occurred via the spacer $-CH_2-CH=CH-CO-NH-CH_2-CH_2-NH-$;
Fig. 4	Shows a cyclic voltammogram of a test site consisting of Au-S-ss-oligo-PQQ (dotted) compared with an identical test site with completely hybridized target (Au-S-ds-oligo-PQQ, solid line);
Fig. 5	Shows a cyclic voltammogram of a test site with completely hybridized target (Au-S-ds-oligo-PQQ) (solid line) compared with a test site with hybridized target that exhibits 2 base-pair mismatches (Au-S-ds-oligo-PQQ with 2 bp mismatches, broken).

PAGE 24, FIRST FULL PARAGRAPH

To prepare the ds oligonucleotide solution, a double-modified 12-bp single-strand oligonucleotide of the sequence 5'-TAGTCGGAAGCA-3' SEQ ID NO: 1 was used, which is esterified with $(HO\text{-}(CH_2)_2\text{-}S)_2$ at the phosphate group of the 3' end to $P\text{-}O\text{-}(CH_2)_2\text{-}S\text{-}S\text{-}(CH_2)_2\text{-}OH$. At the 5' end, the terminal base of the oligonucleotide, thymine, is modified at the C-5 carbon with $-CH{=}CH\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}NH_2$. A $2x10^{-4}$ molar solution of this oligonucleotide in the hybridization buffer (10 mM Tris, 1 mM EDTA, pH 7.5 with 0.7 molar addition of TEATFB, see abbreviations) was hybridized with a $2x10^{-4}$ molar solution of the (unmodified) complementary strand in the hybridization buffer at room temperature for approx. 2 hours (hybridization step). During a reaction time of approx. 12-24 h, the disulfide spacer $P\text{-}O\text{-}(CH_2)_2\text{-}S\text{-}S\text{-}(CH_2)_2\text{-}OH$ of the oligonucleotide was homolytically cleaved. In this process, the spacer forms a covalent Au-S bond with the Au atoms of the surface, thus causing to a 1:1 coadsorption of the ds-oligonucleotide and the 2-hydroxy-mercaptoethanol.

PAGE 24, LAST FULL PARAGRAPH-PAGE 25 TOP PARAGRAPH

Example 2: Producing the Au-S-ss-oligo-PQQ oligonucleotide electrode. Analogously to the production of the Au-S-ds-oligo-PQQ system, the support surface is derivatized with modified single-strand oligonucleotide, dispensing with only the hybridization of the modified oligonucleotide of the sequence 5'-TAGTCGGAAGCA-3' SEQ ID NO: 1 with its complementary strand and, in the incubation step, using only the double-modified 12-bp single-strand probe oligonucleotide (see Example 1) in the form of a 1×10^{-4} molar solution in water and in the presence of 10^{-2} molar Tris, 10^{-3} molar EDTA and 0.7 molar TEATFB (or 1 molar NaCl) at pH 7.5. The redox step was carried out as indicated in Example 1.

PAGE 25, SECOND FULL PARAGRAPH

Example 3: Producing the Au-S-ds-oligo-PQQ oligonucleotide electrode having 2 bp mismatches. The production of a support surface derivatized with modified double-strand oligonucleotide was carried out analogously to the production of the Au-S-ds-oligo-PQQ system, but only in hybridizing the modified oligonucleotide of the sequence 5'-TAGTCGGAAGCA-3' SEQ ID NO: 1 was a complementary strand used (sequence: 5'-ATCAG***AT***TTCGT-3') SEQ ID NO: 2, in which bases no. 6 and 7 (counted from the 5' end), which are actually complementary, were modified from C to ***A*** or from C to ***T*** to introduce two base-pair mismatches.

SEQUENCE LISTING

<110> Hartwich, Gerhard

Heller, Adam

<120> METHOD FOR THE ELECTROCHEMICAL DETECTION OF NUCLEIC

ACID OLIGOMER HYBRIDS

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1 5 10

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	:	Gerhard HARTWICH and Adam HELLER
Serial no.	:	
For	:	METHOD FOR THE ELECTROCHEMICAL DETECTION OF NUCLEIC ACID OLIGOMER HYBRIDS
Docket	:	PATKRI P01AUS

BOX PCT
The Commissioner of Patents and Trademarks
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Dear Sir:

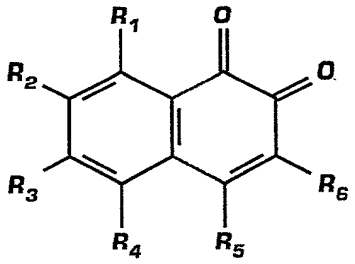
By way of preliminary amendment, please amend the above identified application as set forth below.

In the Claims:

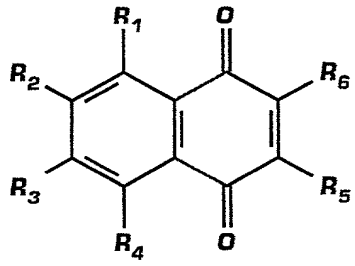
Please cancel original claims 1-28, as well as any Chapter II amended claims, in favor of new claims 29-83 as follows.

Claims

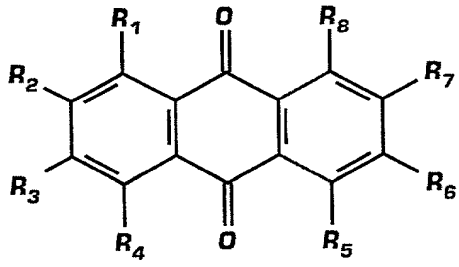
29. (NEW) A nucleic acid oligomer modified by attaching a redox-active substance, characterized in that the redox-active substance is a compound having a predominantly planar p-π-orbital system, namely a 1,2-naphthoquinone of the general structure



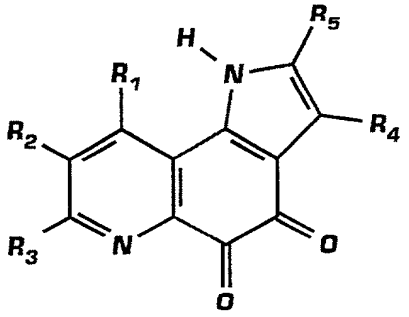
or a 1,4-naphthoquinone of the general structure



or a 9,10-anthraquinone of the general structure

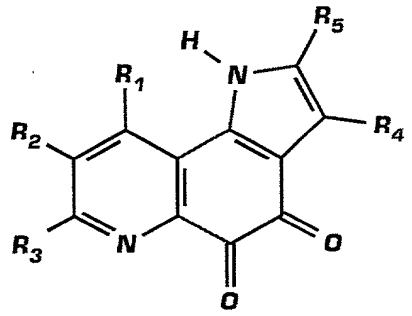


or a pyrrolo-quinoline quinone of the general structure



wherein R_1 to R_8 are, independently of one another, H or any alkyl, alkenyl, alkinyl, heteroalkyl, heteroalkenyl, or heteroalkinyl substituents.

30. (NEW) The modified nucleic acid oligomer according to claim 29 wherein the redox-active substance is a pyrrolo-quinoline quinone of the general structure



wherein R_2, R_4 = H and R_1, R_3, R_5 = COOH.

31. (NEW) The modified nucleic acid oligomer according to claim 29, wherein the redox-active substance is covalently attached to one of the phosphoric-acid, carboxylic-acid, or amine moieties, to one of the sugar moieties, or to one of the bases of the nucleic acid oligomer, especially to a terminal moiety of the nucleic acid oligomer.

32. (NEW) The modified nucleic acid oligomer according to claim 29, wherein the redox-active substance is covalently attached to a branched or linear molecular moiety of any composition and chain length and the branched or linear molecular moiety is attached to one of the phosphoric-acid, carboxylic-acid, or amine moieties, to one of the sugar moieties, or to one of the bases of the nucleic acid oligomer, especially to a terminal moiety of the nucleic acid oligomer.

33. (NEW) The modified nucleic acid oligomer according to claim 32 wherein the redox-active substance is covalently attached to a branched or linear molecular moiety whose shortest continuous link between the joined structures comprises 1 to 14 atoms.

34. (NEW) The modified nucleic acid oligomer according to claim 29, wherein the modified nucleic acid oligomer can sequence-specifically bind single-strand DNA, RNA, and/or PNA.

35. (NEW) The modified nucleic acid oligomer according to claim 34 wherein the modified nucleic acid oligomer is a deoxyribonucleic acid oligomer, a ribonucleic acid oligomer, a peptide nucleic acid oligomer, or a nucleic acid oligomer having a structurally analogous backbone.

36. (NEW) The method of producing a modified nucleic acid oligomer according to claim 29 , characterized in that the redox-active substance is bound to a nucleic acid oligomer, the attachment occurring at a phosphoric-acid or carboxylic-acid group of the nucleic acid oligomer by means of amidation with a (primary or secondary) amino group of the redox-active substance, by means of esterification with a (primary, secondary, or tertiary) alcohol group of the redox-active substance, by means of thioester formation with a (primary, secondary, or tertiary) thioalcohol group of the redox-active substance, or by means of condensation of an amine group of the nucleic acid oligomer with an aldehyde group of the redox-active substance.

37. (NEW) The method of producing a modified nucleic acid oligomer according to claim 32 , characterized in that the redox-active substance is covalently attached to a branched or linear molecular moiety of any composition and chain length, the attachment occurring at a phosphoric-acid or carboxylic-acid group of the branched or linear molecular moiety by means of amidation with a (primary or secondary) amino group of the redox-active substance, by means of esterification with a (primary, secondary, or tertiary) alcohol group of the redox-active substance, by means of thioester formation with a (primary, secondary, or tertiary) thioalcohol group of the redox-active substance, or by means of condensation of an amine group of the branched or linear molecular moiety with an aldehyde group of the redox-active substance.

38. (NEW) A modified conductive surface, characterized in that one or more kinds of modified nucleic acid oligomers according to claim 29 are attached to a conductive surface.

39. (NEW) The modified conductive surface according to claim 38, wherein the surface consists of a metal or a metal alloy, especially a metal selected from the group platinum, palladium, gold, cadmium, mercury, nickel, zinc, carbon, silver, copper, iron, lead, aluminum, manganese, and their compounds.

40. (NEW) The modified conductive surface according to claim 38, wherein the surface consists of a semiconductor, especially a semiconductor selected from the group carbon, silicon, germanium, and α-tin.

41. (NEW) The modified conductive surface according to claim 38, wherein the surface consists of a binary compound of the elements of groups 14 and 16, a binary compound of the elements of groups 13 and 15, a binary compound of the elements of groups 15 and 16, or a binary compound of the elements of groups 11 and 17, especially a Cu(I)-halide or an Ag(I)-halide.

42. (NEW) The modified conductive surface according to claim 38, wherein the surface consists of a ternary compound of the elements of groups 11, 13, and 16, or a ternary compound of the elements of groups 12, 13, and 16.

43. (NEW) The modified conductive surface according to one of claim 38, wherein the modified nucleic acid oligomers are attached to the conductive surface covalently or by means of physisorption.

44. (NEW) The modified conductive surface according to claim 43, wherein one of the phosphoric-acid, carboxylic-acid, or amine moieties, one of the sugar moieties, or one of the bases of the nucleic acid oligomer is attached to the conductive surface covalently or by means of physisorption, especially to a terminal moiety of the nucleic acid oligomer.

45. (NEW) The modified conductive surface according to claim 38, wherein branched or linear molecular moieties of any composition and chain length are attached to the conductive surface, covalently or by means of physisorption, and the modified nucleic acid oligomers are covalently attached to these molecular moieties.

46. (NEW) The modified conductive surface according to claim 55, wherein the branched or linear molecular moiety comprises a shortest continuous link of 1 to 14 atoms between the joined structures.

47. (NEW) The modified conductive surface according to claim 45, wherein the branched or linear molecular moiety is covalently bound to one of the phosphoric-acid, carboxylic-acid, or amine moieties, to one of the sugar moieties, or to one of the bases of the nucleic acid oligomer, especially to a terminal moiety of the nucleic acid oligomer.

48. (NEW) A method of producing a modified conductive surface according to claim 38, wherein one or more kinds of modified nucleic acid oligomers according to claims 1 to 7 are applied to a conductive surface.

49. (NEW) The method of producing a modified conductive surface according to claim 38, wherein one or more kinds of nucleic acid oligomers are bound to a conductive surface and only the nucleic acid oligomers bound to the conductive surface are modified by attaching a redox-active substance to the nucleic acid oligomers.

50. (NEW) The method of producing a modified conductive surface according to claim 49, wherein the attachment of the redox-active substance to the nucleic acid oligomer occurs by means of reacting the redox-active substance with a phosphoric-acid moiety, a sugar moiety, or one of the bases of the nucleic acid oligomer, especially by means of reaction with a terminal moiety of the nucleic acid oligomer.

51. (NEW) The method of producing a modified conductive surface according to claim 49, wherein the redox-active substance is covalently attached to a branched or linear molecular moiety of any composition and chain length and the branched or linear molecular moiety is attached to one of the phosphoric-acid, carboxylic-acid, or amine moieties, to one of the sugar moieties, or to one of the bases of the nucleic acid oligomer, especially to a terminal moiety of the nucleic acid oligomer.

52. (NEW) The method of producing a modified conductive surface according to claim 48, wherein the nucleic acid oligomer or the modified nucleic acid

oligomer is hybridized with the nucleic acid oligomer strand complementary to it and applied to the conductive surface in the form of the double-strand hybrid.

53. (NEW) The method of producing a modified conductive surface according to claim 48, wherein the nucleic acid oligomer or the modified nucleic acid oligomer is applied to the conductive surface in the presence of further chemical compounds that are likewise attached to the conductive surface.

54. (NEW) A method of electrochemically detecting nucleic acid oligomer hybridization events, characterized in that a conductive surface as defined in claim 38, is brought into contact with nucleic acid oligomers and, thereafter, detection of the change in the electrical communication between the redox-active moiety and the respective conductive surface resulting from the hybridization of the nucleic acid oligomers with the modified nucleic acid oligomers occurs.

55. (NEW) The method according to claim 54, wherein detection occurs by means of cyclic voltammetry, amperometry, or conductivity measurement.

56. (NEW) A method of producing a modified conductive surface, wherein a nucleic acid oligomer or a nucleic acid oligomer modified by attaching a redox-active substance that is selectively oxidizable and reducible at a potential φ with 2.0 V $\geq \varphi \geq$ - 2.0 V, measured against normal hydrogen electrode, is hybridized with the nucleic acid oligomer strand complementary to it and applied to a conductive surface in the form of the double-strand hybrid.

57. (NEW) The method according to claim 56, wherein the double-strand hybrid is thermally dehybridized following application to the conductive surface.

58. (NEW) The method according to claim 56, wherein the double-strand hybrid is applied to the conductive surface in the presence of further chemical compounds that are likewise attached to the conductive surface.

59. (NEW) A method of producing a modified conductive surface, wherein a nucleic acid oligomer or a nucleic acid oligomer modified by attaching a redox-active substance that is selectively oxidizable and reducible at a potential φ with 2.0 V $\geq \varphi \geq$ - 2.0 V, measured against normal hydrogen electrode, is applied to the conductive surface in the presence of further chemical compounds that are likewise attached to the conductive surface.

60. (NEW) A method of producing a modified conductive surface wherein a nucleic acid oligomer or a nucleic acid oligomer modified by attaching a redox-active substance that is selectively oxidizable and reducible at a potential φ, with 2.0 V $\geq \varphi \geq$ - 2.0 V, measured against normal hydrogen electrode, is applied to the conductive surface in a buffer with no conducting salt added, to reduce electrostatic shielding of the nucleic acid oligomer, and thereafter, further chemical compounds that are likewise attached to the conductive surface are applied to the conductive surface.

61. (NEW) The method according to claim 30, wherein the chemical compounds are alkyl, alkenyl, alkinyl, heteroalkyl, heteroalkenyl, or heteroalkinyl chains.

62. (NEW) The method according to claim 33, wherein the alkyl, alkenyl, alkinyl, heteroalkyl, heteroalkenyl, or heteroalkinyl chains have a chain length of 1 to 20 atoms.

63. (NEW) The method according to claim 34, wherein the alkyl, alkenyl, alkinyl, heteroalkyl, heteroalkenyl, or heteroalkinyl chains have a chain length of 1 to 14 atoms.

64. (NEW) The method according to claim 56, wherein the nucleic acid oligomers or the modified nucleic acid oligomers are attached to the conductive surface covalently or by means of physisorption.

65. (NEW) The method according to claim 36, wherein one of the phosphoric-acid, carboxylic-acid, or amine moieties, one of the sugar moieties, or one of the bases of the nucleic acid oligomer or of the modified nucleic acid oligomer is attached to the conductive surface covalently or by means of physisorption, especially to a terminal moiety of the nucleic acid oligomer or of the modified nucleic acid oligomer.

66. (NEW) The method according to claim 56, wherein the nucleic acid oligomers or the modified nucleic acid oligomers are covalently attached to branched or linear molecular moieties of any composition and chain length and these molecular moieties are attached to the conductive surface covalently or by means of physisorption.

67. (NEW) The method according to claim 38, wherein the chain length of the branched or linear molecular moiety is 1 to 14 atoms.

68. (NEW) The method according to claim 38, wherein the branched or linear molecular moiety is covalently bound to one of the phosphoric-acid, carboxylic-acid, or amine moieties, to one of the sugar moieties, or to one of the bases of the nucleic acid oligomer, especially to a terminal moiety of the nucleic acid oligomer.

69. (NEW) The method according to one of claim 30, wherein the chain length of the further chemical compound and the chain length of the branched or linear molecular moiety differ by a maximum of 8 atoms.

70. (NEW) The method according to claim 41, wherein the chemical compound and the branched or linear molecular moiety have the same chain length.

71. (NEW) The method according to claim 56, wherein one or more kinds of nucleic acid oligomers in the form of the double-strand hybrid are bound to a conductive surface and only the nucleic acid oligomers bound to the conductive surface are modified by attaching a redox-active substance to the nucleic acid oligomers.

72. (NEW) The method according to claim 43, wherein the attachment of the redox-active substance to the nucleic acid oligomer occurs by means of reacting the redox-active substance with a phosphoric-acid moiety, a sugar moiety, or one of the bases of the nucleic acid oligomer, especially by means of reaction with a terminal moiety of the nucleic acid oligomer.

73. (NEW) The method according to claim 44, wherein the redox-active substance is covalently attached to a branched or linear molecular moiety of any composition and chain length and the branched or linear molecular moiety is attached to one of the phosphoric-acid, carboxylic-acid, or amine moieties, to one of the sugar moieties, or to one of the bases of the nucleic acid oligomer, especially to a terminal moiety of the nucleic acid oligomer.

74. (NEW) The method according to claim 56, wherein the redox-active substance is a dye, especially a flavine derivative, a porphyrin derivative, a chlorophyll derivative, or a bacteriochlorophyll derivative.

75. (NEW) The method according to claim 56, wherein the redox-active substance is a quinone, especially a pyrrolo-quinoline quinone (PQQ), a 1,4-benzoquinone, a 1,2-naphthoquinone, a 1,4-naphthoquinone, or a 9,10-anthraquinone.

76. (NEW) The method according to claim 46, wherein the redox-active substance is covalently attached to one of the phosphoric-acid, carboxylic-acid, or amine moieties, to one of the sugar moieties, or to one of the bases of the nucleic acid oligomer, especially to a terminal moiety of the nucleic acid oligomer.

77. (NEW) The method according to claim 46, wherein the redox-active substance is covalently attached to a branched or linear molecular moiety of any composition and chain length and the branched or linear molecular moiety is attached to one of the phosphoric-acid, carboxylic-acid, or amine moieties, to one of the sugar moieties, or to one of the bases of the nucleic acid oligomer, especially to a terminal moiety of the nucleic acid oligomer.

78. (NEW) The method according to claim 49, wherein the redox-active substance is covalently attached to a branched or linear molecular moiety whose shortest continuous link between joined structures comprises 1 to 14 atoms.

79. (NEW) The method according to claim 56, wherein the modified nucleic acid oligomer is a deoxyribonucleic acid oligomer, a ribonucleic acid oligomer, a peptide nucleic acid oligomer, or a nucleic acid oligomer having a structurally analogous backbone.

80. (NEW) The method according to claim 56, wherein the conductive surface consists of a metal or a metal alloy, especially a metal selected from the group platinum, palladium, gold, cadmium, mercury, nickel, zinc, carbon, silver, copper, iron, lead, aluminum, manganese, and their compounds.

81. (NEW) The method according to claim 56, wherein the conductive surface consists of a semiconductor, especially a semiconductor selected from the group carbon, silicon, germanium, and α-tin.

82. (NEW) The method according to claim 56, wherein the conductive surface consists of a binary compound of the elements of groups 14 and 16, a binary compound of the elements of groups 13 and 15, a binary compound of the elements of groups 15 and 16, or a binary compound of the elements of groups 11 and 17, especially a Cu(I)-halide or an Ag(I)-halide.

83. (NEW) The method according to claim 56, wherein the conductive surface consists of a ternary compound of the elements of groups 11, 13, and 16, or a ternary compound of the elements of groups 12, 13, and 16.

REMARKS

Please enter the above before consideration of this application. With respect to the above newly entered claims, please note that the subject matter of the Chapter II amended claims is editorially revised and rewritten to bring that subject matter into conformity with the United States claim format.

In the event that there are any fee deficiencies or additional fees are payable, please charge the same or credit any overpayment to our Deposit Account (Account No. 04-0213).

Respectfully submitted,

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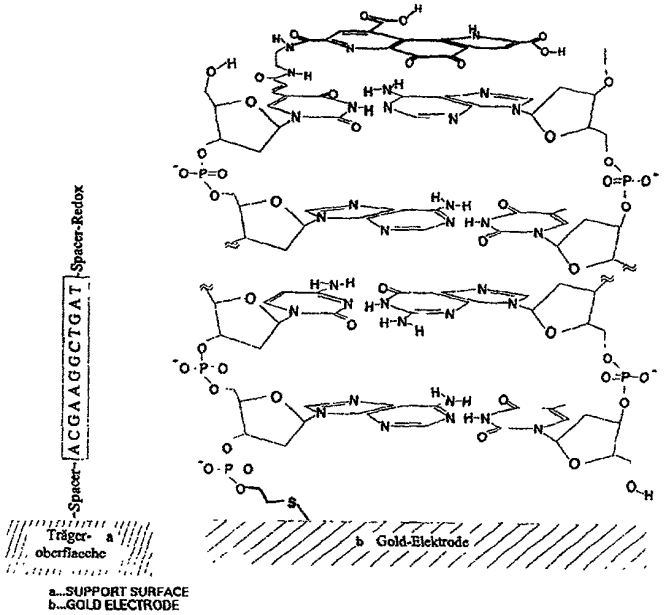
(54) Title: METHOD FOR THE ELECTROCHEMICAL DETECTION OF NUCLEIC ACID OLIGOMER HYBRIDS

(54) Bezeichnung: VERFAHREN ZUR ELEKTROCHEMISCHEN DETEKTION VON NUKLEINSÄUREOLIGOMERHYBRIDEN

(57) Abstract

The invention relates to a method for the electrochemical detection of sequence–specific nucleic acid oligomer hybridization events. To this end single DNA/RNA/PNA oligomer strands which at one end are covalently joined to a support surface and at the other, free end, covalently linked to a redox pair, are used as hybridization matrix (probe). As a result of treatment with the olignucleotide solution (target) to be examined, the electric communication between the conductive support surface and the redox pair bridged by the single–strand oligonucleotide, which communication initially is either absent or very weak, is modified. In case of hybridization, electric communication between the surface support and the redox pair, which is now bridged by a hybridized double–strand oligonucleotide, is increased. This permits the detection of a hybridization event by electrochemical methods such as cyclic voltametry, amperometry or conductivity measurement.



(57) Zusammenfassung

Die vorliegende Erfindung betrifft ein Verfahren zur elektrochemischen Detektion von sequenzspezifischen Nukleinsäure–Oligomer–Hybridisierungsereignissen. Dabei dienen DNA–/RNA–/PNA–Oligomer–Einzelstränge, die mit einem Ende kovalent auf einer Trägeroberfläche angebunden und am anderen, freien Ende kovalent mit einem Redoxpaar verknüpft sind, als Hybridisierungsmatrix ("Sonde"). Durch Behandlung mit der zu untersuchenden Oligonukleotid–Lösung ("Target") wird die ursprünglich nicht oder nur schwach vorhandene elektrische Kommunikation zwischen der leitfähigen Trägeroberfläche und dem über Einzelstrang–Oligonukleotid verbrückten Redoxpaar verändert. Im Falle der Hybridisierung wird die elektrische Kommunikation zwischen der Trägeroberfläche und dem nunmehr über hybridisiertes Doppelstrang–Oligonukleotid verbrückten Redoxpaar verstärkt. Somit wird die Detektion eines Hybridisierungsereignisses durch elektrochemische Verfah

Method of Electrochemically Detecting Nucleic Acid Oligomer Hybrids

Field of the Invention

The present invention is directed to a modified nucleic acid oligomer, as well as a method of electrochemically detecting sequence-specific nucleic acid oligomer hybridization events.

Background of the Invention

Generally, gel-electrophoretic methods with autoradiographical or optical detection are used for DNA and RNA sequence analysis, e.g. in disease diagnosis, toxicological test procedures, genetic research and development, as well as in the agrarian and pharmaceutical sectors.

To illustrate the most significant gel-electrophoretic method with optical detection (Sanger method), Figure 1b shows a DNA fragment with primer. In the Sanger method, a DNA-containing solution is divided into four samples and the primer of each sample is covalently modified with a fluorescent dye that emits at a distinct wavelength. As illustrated in Figure 1b, deoxyribonucleoside triphosphate of bases A (adenine), T (thymine), C (cytosine), and G (guanine), i.e. dATP, dTTP, dCTP, and dGTP, are added to each sample to enzymatically replicate the single strand, starting at the primer, by means of DNA polymerase I. In addition to the four deoxyribonucleoside triphosphates, each reaction mixture also contains sufficient 2',3'-dideoxy analog (Figure 1a) of one of these nucleoside triphosphates as a blocking base (one of each of the four possible blocking bases per sample) to terminate replication at all possible binding sites. After combining the four samples, all lengths of replicated DNA fragments having blocking-base-specific fluorescence result and can be gel-electrophoretically sorted according to length and characterized using fluorescent spectroscopy (Figure 1c).

Another optical detection method is based on the accumulation of fluorescent dyes such as e.g. ethidium bromide on oligonucleotides. The fluorescence of such dyes increases in comparison with the free solution of the dye by about 20-fold when they accumulate on double-stranded DNA or RNA and can therefore be used to detect hybridized DNA or RNA.

In radiolabeling, ^{32}P is built into the phosphate skeleton of the oligonucleotides, with ^{32}P usually being added to the 5'-hydroxyl end by means of polynucleotide kinase. Thereafter, the labeled DNA is preferably cleaved, under defined conditions, at one of each of the four nucleotide types, such that an average of one cleavage per chain results. Thus, for a given base type, there are present in the reaction mixture chains extending from the ^{32}P-label to the position of that base (if there are multiple appearances of the base, chains of varying lengths will result accordingly). The four fragment mixtures are then gel-electrophoretically separated on four lanes. Thereafter, an autoradiogram of the gel is prepared, from which the sequence can be directly read.

Some years ago, a further method of DNA sequencing was developed on the basis of optical (or autoradiographical) detection, namely sequencing by means of oligomer hybridization (cf. e.g. Drmanac et al., Genomics 4, (1989), pp. 114-128 or Bains et al., Theor. Biol. 135, (1988), pp.303-307). In this method, a complete set of short oligonucleotides, or oligomers (probe oligonucleotides), e.g. all 65,536 possible combinations of the bases A, T, C, and G of an oligonucleotide octamer are bound to a support. The attachment occurs in an ordered grid consisting of 65,536 test sites, with each larger amount of an oligonucleotide combination defining one test site, and the position of each individual test site (oligonucleotide combination) is known. On such a hybridization matrix, the oligomer chip, a DNA fragment whose sequence is to be determined, the target, is labeled with fluorescent dye (or ^{32}P) and hybridized under conditions that allow only one specific double-strand formation. In this way, the target DNA fragment attaches only to the oligomers (in this example to the octamers) whose complementary sequence corresponds exactly to a portion (an octamer) of its own sequence. Thus, all of the oligomer sequences (octamer sequences) present in the fragment are determined by means of optical (or autoradiographical) detection of the binding position of the hybridized DNA fragment. Due to the overlapping of neighboring oligomer sequences, the continuous sequence of the DNA fragment can be determined using suitable mathematical algorithms. The advantages of this method lie in, among other things, the miniaturization of the sequencing and thus in the enormous amount of data that can be simultaneously captured in one operation. In addition, primer and gel-electrophoretic separation of the DNA fragments can be dispensed with. This principle is demonstrated by example in Figure 2 for a 13-base-long DNA fragment.

The use of radioactive labels in DNA/RNA sequencing is associated with several disadvantages, such as e.g. elaborate, legally required safety precautions in dealing with radioactive materials, radiation, spatially limited resolution capacity (maximum 1 mm^2) and sensitivity that is only high when the radiation of the radioactive fragments act on an X-ray film for an appropriately long time (hours to days). Although the spatial resolution can be increased by means of additional hardware and software, and the detection time can be decreased by means of β-scanners, both of these involve considerable additional costs.

Some of the fluorescent dyes that are commonly used to label the DNA (e.g. ethidium bromide) are mutagenic and require appropriate safety precautions, as does the use of autoradiography. In nearly every case, the use of optical detection requires the use of one or more laser systems, and thus experienced personnel and appropriate safety precautions. The actual detection of the fluorescence requires additional hardware such as e.g. optical components for amplification and, in the case of varying stimulation and query wavelengths as in the Sanger method, a control system. Thus, depending on the stimulation wavelengths required and the detection performance desired, considerable investment costs may result. In sequencing by means of hybridization on the oligomer chip, detection is even more costly because, in addition to the stimulation system, high-resolution CCD cameras (charge coupled device cameras) are needed for 2-dimensionally detecting the fluorescent spots.

Thus, although there are quantitative and extremely sensitive methods for DNA/RNA sequencing, these methods are time consuming, require elaborate sample preparation and expensive equipment, and are generally not available as portable systems.

Description of the Invention

Therefore, it is the object of the present invention to create for detecting nucleic acid oligomer hybrids an apparatus and a method that do not exhibit the disadvantages of the state of the art.

According to the present invention, this object is solved by the modified oligonucleotide according to independent claim 1, by the method of producing a modified oligonucleotide according to independent claims 9 and 10, by the modified conductive surface according to independent claim 11, the method of producing a

modified conductive surface according to independent claim 21, and a method of electrochemically detecting oligomer hybridization events according to independent claim 27.

The following abbreviations and terms are used herein:

Genetics	
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
PNA	peptide nucleic acid (Synthetic DNA or RNA in which the sugar-phosphate moiety is replaced by an amino acid. If the sugar-phosphate moiety is replaced by the -NH-$(CH_2)_2$-N($COCH_2$-base)-CH_2CO- moiety, PNA will hybridize with DNA.)
A	adenine
G	guanine
C	cytosine
T	thymine
base	A, G, T, or C
bp	base pair
nucleic acid	At least two covalently joined nucleotides or at least two covalently joined pyrimidine (e.g. cytosine, thymine, or uracil) or purine bases (e.g. adenine or guanine). The term nucleic acid refers to any backbone of the covalently joined pyrimidine or purine bases, such as e.g. to the sugar-phosphate backbone of DNA, cDNA, or RNA, to a peptide backbone of PNA, or to analogous structures (e.g. a phosphoramide, thiophosphate, or dithiophosphate backbone). The essential feature of a nucleic acid according to the present invention is that it can sequence-specifically bind naturally occurring cDNA or RNA.
nucleic acid oligomer	Nucleic acid of base length that is not further specified (e.g. nucleic acid octamer: a nucleic acid having any backbone in which 8 pyrimidine or purin bases are covalently bound to

	one another).
oligomer	Equivalent to nucleic acid oligomer.
oligonucleotide	Equivalent to oligomer or nucleic acid oligomer, thus e.g. a DNA, PNA, or RNA fragment of base length that is not further specified.
oligo	Abbreviation for oligonucleotide.
dATP	Deoxyribonucleoside triphosphate of A (DNA moiety with the A base and two further phosphates to build a longer DNA fragment or oligonucleotide).
dGTP	Deoxyribonucleoside triphosphate of G (DNA moiety with the G base and two further phosphates to build a longer DNA fragment or oligonucleotide).
dCTP	Deoxyribonucleoside triphosphate of C (DNA moiety with the C base and two further phosphates to build a longer DNA fragment or oligonucleotide).
dTTP	Deoxyribonucleoside triphosphate of T (DNA moiety with the T base and two further phosphates to build a longer DNA fragment or oligonucleotide).
primer	Initial complementary fragment of an oligonucleotide, with the base length of the primer being only approx. 4-8 bases. Serves as the starting point for enzymatic replication of an oligonucleotide.
mismatch	To form the Watson Crick double-stranded oligonucleotide structure, the two single strands hybridize in such a way that the A (or C) base of one strand forms hydrogen bonds with the T (or G) base of the other strand (in RNA, T is replaced by uracil). Any other base pairing does not form hydrogen bonds, distorts the structure, and is referred to as a "mismatch."
ds	double strand
ss	single strand
Chemical Substances/Groups	
R	A substituent or side chain of any organic residue not further

	specified.
redox	redox-active substance
alkyl	The term "alkyl" refers to a saturated hydrocarbon radical that is straight-chained or branched (e.g. ethyl, isopropyl, or 2,5-dimethylhexyl, etc.). When "alkyl" is used to indicate a linker or spacer, the term refers to a group having two available valences for covalent linkage (e.g. $-CH_2CH_2-$, $-CH_2CH_2CH_2-$, or $-CH_2C(CH_3)_2CH_2CH_2C(CH_3)_2CH_2-$, etc.). Alkyl groups preferred as substituents or side chains R are those of chain length 1-30 (longest continuous chain of atoms bound to one another). Alkyl groups preferred as linkers or spacers are those of chain length 1-20, especially of chain length of 1-14, the chain length representing the shortest continuous link between linker or spacer-joined structures.
alkenyl	Alkyl groups in which one or more of the C-C single bonds are replaced by C=C double bonds.
alkinyl	Alkyl or alkenyl groups in which one or more of the C-C single or C=C double bonds are replaced by C≡C triple bonds.
heteroalkyl	Alkyl groups in which one or more of the C-H bonds or C-C single bonds are replaced by C-N, C=N, C-P, C=P, C-O, C=O, C-S, or C=S bonds.
heteroalkenyl	Alkenyl groups in which one or more C-H bonds, C-C single, or C=C double bonds are replaced by C-N, C=N, C-P, C=P, C-O, C=O, C-S, or C=S bonds.
heteroalkinyl	Alkinyl groups in which one or more of the C-H bonds, C-C single, C=C double, or C≡C triple bonds are replaced by C-N, C=N, C-P, C=P, C-O, C=O, C-S, or C=S bonds.
linker	A molecular link between two molecules or between a surface atom, surface molecule, or surface molecule group and another molecule. Linkers can usually be purchased in the form of alkyl, alkenyl, alkinyl, heteroalkyl, heteroalkenyl, or heteroalkinyl chains, the chain being derivatized in two places with (identical or different) reactive groups. These groups form a covalent chemical bond in simple/known chemical reactions with the appropriate reaction partner. The reactive

	groups may also be photoactivatable, i.e. the reactive groups are activated only by light of a specific or random wavelength. Preferred linkers are those of chain length of 1-20, especially of chain length of 1-14, the chain length representing here the shortest continuous link between the structures to be joined, thus between the two molecules or between a surface atom, surface molecule, or surface molecule group and another molecule.
spacer	A linker that is covalently attached via the reactive groups to one or both of the structures to be joined (see linker). Preferred spacers are those of chain length 1-20, especially of chain length 1-14, the chain length representing the shortest continuous link between the structures to be joined.
(n x HS-spacer)-oligo	A nucleic acid oligomer to which n thiol functions are each attached via a spacer, where each spacer may have a different chain length (shortest continuous link between the thiol function and the nucleic acid oligomer), especially any chain length between 1 and 14 each. These spacers, in turn, may be bound to various reactive groups that are naturally present on the nucleic acid oligomer or that have been fixed thereto by means of modification, and "n" is any integer, especially a number between 1 and 20.
(n x R-S-S-spacer)-oligo	A nucleic acid oligomer to which n disulfide functions are each attached via a spacer, and any residue R saturates the disulfide function. Each spacer for attaching the disulfide function to the nucleic acid oligomer may have a different chain length (shortest continuous link between the disulfide function and the nucleic acid oligomer), especially any chain length between 1 and 14 each. These spacers, in turn, may be bound to various reactive groups that are naturally present on the nucleic acid oligomer or that have been fixed thereto by means of modification. The placeholder "n" is any integer, especially a number between 1 and 20.
oligo-spacer-S-S-spacer-oligo	Two identical or different nucleic acid oligomers that are joined to each other via a disulfide bridge, the disulfide bridge being attached to the nucleic acid oligomers via any two spacers and the two spacers potentially having differing chain lengths

	(shortest continuous link between the disulfide bridge and the respective nucleic acid oligomer), especially any chain length between 1 and 14 each, and these spacers, in turn, potentially being bound to various reactive groups that are naturally present on the nucleic acid oligomer or that have been fixed thereto by means of modification.
PQQ	pyrroloquinoline quinone; corresponds to 4,5-dihydro-4,5-dioxo-1H-pyrrolo-[2,3-f]-quinoline-2,7,9-tricarboxylic acid)
TEATFB	tetraethylammonium-tetrafluoroborate
sulfo-NHS	N-hydroxysulfosuccinimide
EDC	(3-dimethylaminopropyl)-carbodiimide
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
Tris	trishydroxymethylamino methane
EDTA	ethylenediamine tetraacetate (sodium salt)
cystamine	$(H_2N\text{-}CH_2\text{-}CH_2\text{-}S\text{-})_2$
Modified Surfaces/Electrodes	
mica	Muskovite platelets, a support for the application of thin layers.
Au-S-ss-oligo-PQQ	Gold film on mica having a covalently applied monolayer of derivatized 12-bp single-strand oligonucleotide (sequence: TAGTCGGAAGCA). Here, the terminal phosphate group of the oligonucleotide at the 3' end is esterified with $(HO\text{-}(CH_2)_2\text{-}S)_2$ to $P\text{-}O\text{-}(CH_2)_2\text{-}S\text{-}S\text{-}(CH_2)_2\text{-}OH$, homolytically cleaving the S-S bond and producing one Au-S-R bond each. The terminal thymine base at the 5' end of the oligonucleotide is modified at the C-5 carbon with $\text{-}CH{=}CH\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}NH_2$ and the residue, in turn, is joined via its free amino group with a carboxylic-acid group of the PQQ by means of amidation.
Au-S-ds-oligo-PQQ	Au-S-ss-oligo-PQQ that is hybridized with the oligonucleotide complementary to the ss-oligo (sequence: TAGTCGGAAGCA).
Electrochemistry	

E	The electrode potential on the working electrode.
E_0	Half-wave potential, the potential in the middle between the current maximums for oxidation and reduction of cyclic voltammetrically reversible electrooxidation or reduction.
i	current density (current per cm^2 of electrode surface)
cyclic voltammetry	Recording a current-voltage curve. The potential of a stationary working electrode is changed linearly as a function of time, starting at a potential at which no electrooxidation or reduction occurs, up to a potential at which a species that is solute or adsorbed on the electrode is oxidized or reduced (i.e. current flows). After running through the oxidation or reduction operation, which produces in the current-voltage curve an initially increasing current and, after reaching a maximum, a gradually decreasing current, the direction of the potential feed is reversed. The behavior of the products of electrooxidation or electroreduction is then recorded in reverse run.
amperometry	Recording a current-time curve. Here, the potential of a stationary working electrode is set, e.g. by means of a potential jump, to a potential at which the electrooxidation or reduction of a solute or adsorbed species occurs, and the flowing current is recorded as a function of time.

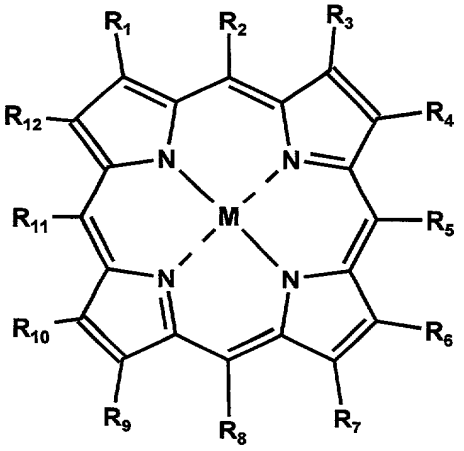
The present invention is directed to a nucleic acid oligomer that is modified by the chemical attachment of a redox-active substance. According to the present invention, the nucleic acid oligomer is a compound consisting of at least two covalently joined nucleotides or at least two covalently joined pyrimidine (e.g. cytosine, thymine, or uracil) or purine bases (e.g. adenine or guanine), preferably a DNA, RNA, or PNA fragment. As used herein, the term nucleic acid refers to any backbone of the covalently joined pyrimidine or purine bases, such as e.g. to the sugar-phosphate backbone of DNA, cDNA, or RNA, to a peptide backbone of PNA, or to analogous backbone structures such as e.g. a thiophosphate, a dithiophosphate, or a phosphoramide backbone. The essential feature of a nucleic acid according to the present invention is that it can sequence-specifically bind naturally occurring cDNA or RNA. The terms "(probe) oligonucleotide," "nucleic acid," and "oligomer" are used as alternatives to the term "nucleic acid oligomer."

The redox-active substance is selectively oxidizable and reducible at a potential φ, where φ satisfies the condition $2.0\ V \geq \varphi \geq -2.0\ V$. The potential refers here to the free, unmodified, redox-active substance in a suitable solvent, measured against normal hydrogen electrode. According to the present invention, the potential range $1.7\ V \geq \varphi \geq -1.7\ V$ is preferred, the range $1.4\ V \geq \varphi \geq -1.2\ V$ being particularly preferred, and the range $0.9\ V \geq \varphi \geq -0.7\ V$, in which the redox-active substances of the application example are reduced and reoxidized, being most particularly preferred. In addition, the present invention is directed to a conductive surface to which a nucleic acid oligomer having an attached redox-active substance is chemically bound, directly or indirectly (via a spacer). Furthermore, the present invention is directed to a method of producing a modified conductive surface, wherein a modified nucleic acid oligomer is applied to a conductive surface. According to a further aspect, the present invention is directed to a method that allows electrochemical detection of molecular structures, especially electrochemical detection of DNA/RNA/PNA fragments in a probe solution by means of sequence-specific nucleic acid oligomer hybridization. Detection of hybridization events by means of electrical signals is a simple and economical method and, in a battery-operated variation of a sequencing device, allows on-site application.

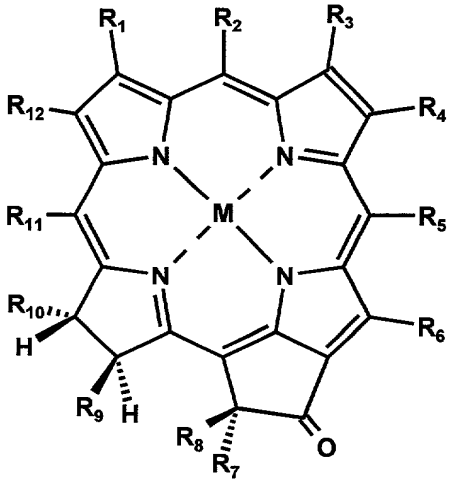
Binding a Redoxactive Moiety to a Nucleic Acid Oligomer

For the method of the present invention, it is necessary to bind a redox-active substance to a nucleic acid oligomer. According to the present invention, any redox-active substance may be used for this purpose as long as it is selectively oxidizable and reducible at a potential φ that satisfies the condition $2.0\ V \geq \varphi \geq -2.0\ V$. The potential refers here to the free, unmodified, redox-active substance in a suitable solvent, measured against normal hydrogen electrode. According to the present invention, the potential range $1.7\ V \geq \varphi \geq -1.7\ V$ is preferred, the range $1.4\ V \geq \varphi \geq -1.2\ V$ is particularly preferred, and the range $0.9\ V \geq \varphi \geq -0.7\ V$, in which the redox-active substances of the application example are reduced and reoxidized, is most particularly preferred. According to the present invention, the term "selectively oxidizable and reducible" is understood to mean a redox reaction, i.e. giving up or taking in an electron, that occurs selectively at the location of the redox-active substance. Thus, in the end, no other part of the nucleic acid oligomer is reduced or oxidized by the potential applied, but rather, exclusively the redox-active substance bound to the nucleic acid oligomer.

According to the present invention, a redox-active substance is understood to mean any molecule that, in the electrochemically accessible potential range of the respective support surface (electrode), can be electrooxidized/electroreduced by applying an external voltage to that electrode. In additon to common organic and anorganic redox-active substances such as e.g. hexacyanoferrates, ferrocenes, acridines, or phtalocyanines, redox-active dyes such as e.g. (metallo-) porphyrins of the general Formula 1, (metallo-) chlorophylls of the general Formula 2, or (metallo-) bacteriochlorophylls of the general Formula 3, (colored) naturally occurring oxidation agents such as e.g. flavines of the general Formula 4, pyridine-nucleotides of the general Formula 5 or pyrrolo-quinoline quinones (PQQ) of the general Formula 6, or other quinones such as e.g. 1,4-benzoquinones of the general Formula 7, 1,2-benzoquinones of the general Formula 8, 1,4-naphthoquinones of the general Formula 9, 1,2-naphthoquinones of the general Formula 10, or 9,10-anthraquinones of the general Formula 11 are particularly suitable for attachment to the probe oligonucleotide.



Formula 1



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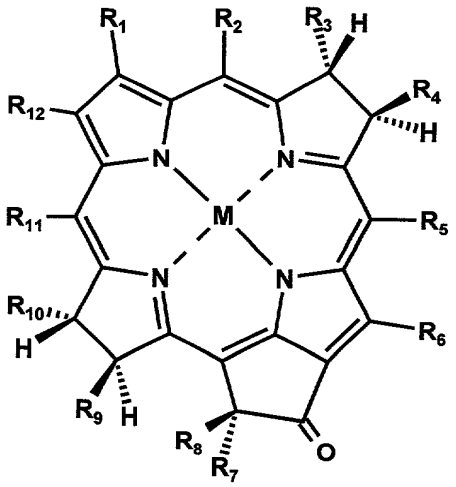
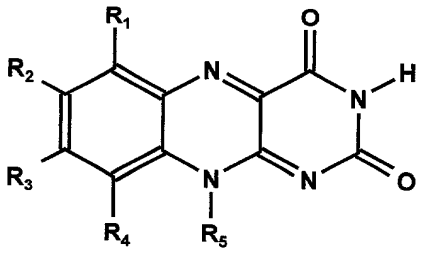


Formula 3

M = 2H, Mg, Zn, Cu, Ni, Pd, Co, Cd, Mn, Fe, Sn, Pt, etc.; R_1 to R_{12} are, independently of one another, H or any alkyl, alkenyl, alkinyl, heteroalkyl, heteroalkenyl, or heteroalkinyl substituents.



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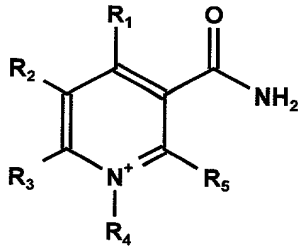

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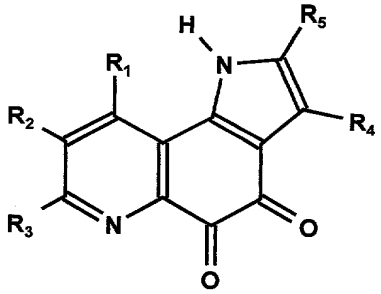

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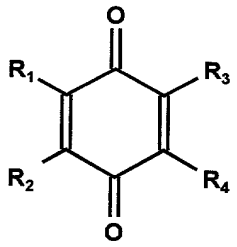

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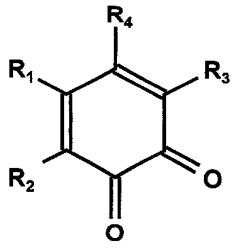

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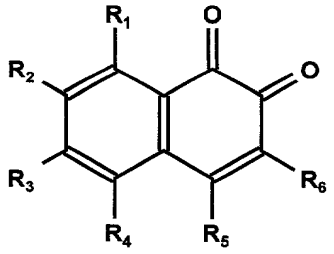

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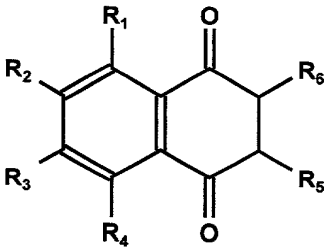

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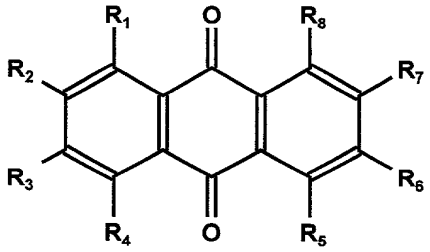


Formula 11

R_1 to R_8 are, independently of one another, H or any alkyl, alkenyl, alkinyl, heteroalkyl, heteroalkenyl, or heteroalkinyl substituents.

According to the present invention, a redox-active substance is covalently bound to an oligonucleotide by means of the oligonucleotide reacting with the redox-active substance. This bond can be achieved in three different ways:

a) The reactive group for forming a bond at the nucleic acid oligomer is a free phosphoric-acid, sugar-C-3-hydroxy, carboxylic-acid, or amine group of the oligonucleotide backbone, especially a group at one of the two ends of the oligonucleotide backbone. The free, terminal phosphoric-acid, sugar-C-3-hydroxy, carboxylic-acid, or amine groups exhibit increased reactivity and thus easily undergo typical reactions such as e.g. amidation with (primary or secondary) amino groups or with acid groups, esterification with (primary, secondary, or tertiary) alcohols or with acid groups, thioester formation with (primary, secondary, or tertiary) thioalcohols or with acid groups, or condensation of amine and aldehyde with subsequent reduction of the resultant CH=N bond to a CH_2-NH bond. The coupling group required for the covalent attachment of the redox-active substance (acid, amine, alcohol, thioalcohol,

or aldehyde function) is either naturally present on the redox-active substance or is obtained by means of chemical modification of the redox-active substance.

b) The nucleic acid oligomer is modified with a reactive group at the oligonucleotide backbone or at a base via a covalently attached molecular moiety (spacer) of any composition and chain length (representing the shortest continuous link between the structures to be joined), especially of chain length 1 to 14. The modification preferably occurs at one of the ends of the oligonucleotide backbone or at a terminal base. Spacers may be e.g. an alkyl, alkenyl, alkinyl, heteroalkyl, heteroalkenyl, or heteroalkinyl substituent. Possible simple reactions for forming the covalent bond between the redox-active substance and the nucleic acid oligomer so modified are, as described under a), amidation from an acid and amino group, esterification from an acid and alcohol group, thioester formation from an acid and thioalcohol group, or condensation of aldehyde and amine with subsequent reduction of the resultant CH=N bond to a CH_2-NH bond.

According to a preferred embodiment, the nucleic acid oligomer is modified using a redox-active substance that exhibits regions having a predominantly planar p-π-orbital system extended in a plane, such as e.g. the PQQ of Example 1 or the quinones of Formulas 5 or 7-12 or the porphinoid structures of Formulas 1-4 or the pyridine nucleotides of the general Formula 6, or derivatives of these redox-active substances. In this case, the spacer via which the redox-active substance is bound to the nucleic acid oligomer can be selected in such a way that the plane of the π-orbitals of the redox-active substance can arrange itself parallel to the p-π-orbitals of the nucleic acid oligomer bases that border on the redox-active substance. This spatial arrangement of the redox-active substance with partially planar p-π-orbitals extended in a plane proves to be particularly favorable.

c) In synthesizing the nucleic acid oligomer, a terminal base will be replaced by the redox-active substance.

According to the present invention, binding the redox-active substance to the oligonucleotide as described under a) and b) may occur before or after binding the oligonucleotide to the conductive surface. The attachment of the redox-active substance to the oligonucleotide bound to the conductive surface then likewise occurs as described under a) and b).

If there are several different oligonucleotide combinations (test sites) on a common surface, it is advantageous to standardize the (covalent) attachment of the redox-active substance to the probe oligonucleotides for the entire surface by the appropriate choice of reactive group at the free probe oligonucleotide ends of the various test sites.

The Conductive Surface

According to the present invention, the term "conductive surface" refers to any support having an electrically conductive surface of any thickness, especially surfaces made of platinum, palladium, gold, cadmium, mercury, nickel, zinc, carbon, silver, copper, iron, lead, aluminum, and manganese. According to the present invention, the terms "electrode" and "conductive (support) surface" are used as alternatives to "conductive surface."

In addition, any doped or non-doped semiconductor surfaces of any thickness may also be used. All semiconductors are useful in the form of pure substances or as mixtures. Examples include, but are not limited to, carbon, silicon, germanium, α tin, and Cu(I) and Ag(I) halides of any crystal structure. Likewise suitable are all binary compounds of any composition and any structure of the elements of groups 14 and 16, of the elements of groups 13 and 15, and of the elements of groups 15 and 16. In addition, ternary compounds of any composition and any structure of the elements of groups 11, 13, and 16 or of the elements of groups 12, 13, and 16 may be used. The designations of the groups of the periodic system refer to the IUPAC recommendation of 1985.

Binding an Oligonucleotide to the Conductive Surface

According to the present invention, a nucleotide is linked directly or via a linker/spacer with the support surface atoms or molecules of a conductive support surface of the kind described above. This bond may be carried out in three different ways:

a) The surface is modified in such a way that a reactive molecule group is accessible. This may occur by means of direct derivatization of the surface molecules, e.g. by means of wet chemical or electrochemical oxidation/reduction.

Thus e.g. the surface of graphite electrodes can be wet-chemically supplied with aldehyde or carboxylic-acid groups by means of oxidation. Electrochemically, it is possible e.g. by means of reduction in the presence of aryl-diazonium salts to couple the corresponding (functionalized, i.e. supplied with a reactive group) aryl radical, or by means of oxidation in the presence of $R'CO_2H$ to couple the (functionalized) R' radical to the graphite electrode surface. An example of direct modification of semiconductor surfaces is the derivatization of silicon surfaces to reactive silanols, i.e. silicon supports having Si-OR" groups on the surface, where both R" and R' are any functionalized organic residue (e.g. alkyl, alkenyl, alkinyl, heteroalkyl, heteroalkenyl, or heteroalkinyl substituent). Alternatively, the entire surface may be modified by covalently attaching a reactive group of a bifunctional linker such that a monomolecular layer consisting of any molecules and containing a reactive group, preferably terminally, results on the surface. The term "bifunctional linker" is understood to mean any molecule of any chain length, especially of chain lengths 2-14, having two identical (homobifunctional) or two different (heterobifunctional) reactive molecule groups.

If several different test sites are to be formed on the surface by making use of the methodology of photolithography, then at least one of the reactive groups of the homobifunctional or heterobifunctional linkers is a photoinducible reactive group, i.e. a group that becomes reactive only upon irradiation with light of a specific or random wavelength. This linker is applied in such a way that the/a photoactivatable reactive group is available after the linker is covalently attached to the surface. The nucleic acid oligomers are covalently attached to the surface so modified and are, themselves, modified with a reactive group, preferably near an end of the nucleic acid oligomer, via a spacer of any composition and chain length, especially of chain length 1-14. The reactive group of the oligonucleotide is a group that reacts directly (or indirectly) with the modified surface to form a covalent bond. In addition, a further reactive group may be bound to the nucleic acid oligomers near its second end, this reactive group, in turn, being attached, as described above, directly or via a spacer of any composition and chain length, especially of chain length 1-14. Furthermore, as an alternative to this further reactive group, the redox-active substance may be attached at this second end of the nucleic acid oligomer.

b) The nucleic acid oligomer to be applied to the conductive surface is modified with one or more reactive groups via a covalently attached spacer of any composition and chain length, especially of chain length 1-14, these reactive groups being located preferably near an end of the nucleic acid oligomer. The reactive groups are groups

that can react directly with the unmodified surface. Some examples are: (i) thiol- (HS-) or disulfide- (S-S-) derivatized nucleic acid oligomer of the general formula (n x HS-spacer)-oligo, (n x R-S-S-spacer)-oligo, or oligo-spacer-S-S-spacer-oligo that react with a gold surface to form gold-sulfur bonds, or (ii) amines that accumulate on platinum or silicon sufaces by means of chemisorption or physisorption. In addition, a further reactive group may be bound to the nucleic acid oligomers near its second end, this reactive group, in turn, being attached, as described above, directly or via a spacer of any composition and chain length, especially of chain length 1-14. Furthermore, as an alternative to this further reactive group, the redox-active substance may be attached at this second end of the oligonucleotide. Particularly nucleic acid oligomers that are modified with several spacer-bridged thiol or disulfide bridges ((n x HS-spacer)-oligo or (n x R-S-S-spacer)-oligo) have the advantage that such nucleic acid oligomers can be applied to the conductive surface at a particular setting angle (angle between the surface normal and the helix axis of a double-stranded helical nucleic acid oligomer or between the surface normal and the axis perpendicular to the base pairs of a double-stranded non-helical nucleic acid oligomer) if the spacers attaching the thiol or disulfide functions to the nucleic acid oligomer possess an increasing or decreasing chain length as viewed from an end of the nucleic acid.

c) Phosphoric-acid, sugar-C-3-hydroxy, carboxylic-acid, or amine groups of the oligonucleotide backbone, especially terminal groups, are used as the reactive group on the probe nucleic acid oligomer. The phosphoric-acid, sugar-C-3-hydroxy, carboxylic-acid, or amine groups exhibit greater reactivity and thus easily undergo typical reactions such as amidation with (primary or secondary) amino or acid groups, esterification with (primary, secondary, or tertiary) alcohols or acid groups, thioester formation with (primary, secondary, or tertiary) thioalcohols or acid groups, or condensation of amine and aldehyde with subsequent reduction of the resultant CH=N bond to a CH_2-NH bond. In this case, the coupling group required for the covalent attachment to the phosphoric-acid, sugar-C-3-hydroxy, carboxylic-acid, or amine group is part of the surface derivatization with a (monomolecular) layer of any molecule length, as described under a) in this section, or the phosphoric-acid, sugar-C-3-hydroxy, carboxylic-acid, or amine group can react directly with the unmodified surface, as described under b) in this section. In addition, a further reactive group may be bound to the oligonucleotides near its second end, this reactive group, in turn, being attached, as described above, directly or via a spacer of any composition and chain length, especially of chain length 1-14. Furthermore, as an alternative to

this further reactive group, the redox-active substance may be attached at this second end of the nucleic acid oligomer.

Alternatively, binding the oligonucleotide to the conductive surface may occur before or after attaching the redox-active substance to the oligonucleotide, or before or after attaching the spacer supplied with a reactive group for binding the redox-active substance. Binding the already modified oligonucleotide to the conductive surface, i.e. binding to the surface after attaching the redox-active substance to the oligonucleotide, or after attaching the spacer supplied with a reactive group for binding the redox-active substance, likewise takes place as described under a) to c) (in the section "Binding an Oligonucleotide to the Conductive Surface").

In producing the test sites, care must be taken when attaching the single-strand oligonucleotides to the surface that a sufficiently large distance remains between the individual oligonucleotides to provide the necessary space for a hybridization with the target oligonucleotide. To this end, two different methods of proceeding, among others, present themselves:

1.) Producing a modified support surface by attaching a hybridized oligonucleotide, i.e. a support surface derivatization with hybridized probe oligonucleotide instead of with single-strand probe oligonucleotide. The oligonucleotide strand used for hybridization is unmodified (the surface attachment is carried out as described under a) - c) in the section "Binding an Oligonucleotide to the Conductive Surface"). Thereafter, the hybridized oligonucleotide double strand is thermally dehybridized, thus producing a single-strand-oligonucleotide-modified support surface having greater distance between the probe oligonucleotides.

2.) Producing a modified support surface by attaching a single-strand or double-strand oligonucleotide, and adding during support surface derivatization a suitable monofunctional linker that, in addition to the single-strand or double-strand oligonucleotide, is also bound to the surface (the surface attachment is carried out as described under a) - c) in the section "Binding an Oligonucleotide to the Conductive Surface"). According to the present invention, the monofunctional linker has a chain length that is identical to the chain length of the spacer between the support surface and the oligonucleotide, or that differs by a maximum of eight chain atoms. If double-strand oligonucleotide is used for support-surface derivatization, the hybridized oligonucleotide double strand is thermally dehybridized after attaching the double-strand oligonucleotide and the linker to the support surface, as described under 1.)

above. By simultaneously attaching a linker to the surface, the distance between the single-strand or double-strand nucleic acid oligomers that are likewise bound to the surface is increased. If a double-strand nucleic acid oligomer is used, this effect is amplified further by the subsequent thermal dehybridization.

Method of Electrochemically Detecting Nucleic Acid Oligomer Hybrids

Advantageously, according to the method of electrochemical detection, several probe oligonucleotides varying in sequence, ideally all necessary combinations of the nucleic acid oligomer, are applied to an oligomer chip or DNA chip to reliably detect the sequence of any target oligomer or of a (fragmented) target DNA, or to seek and sequence-specifically detect mutations in the target. To this end, the support surface atoms or molecules of a defined area (a test site) are linked with DNA/RNA/PNA oligonucleotides of known but random sequence on a conductive support surface, as described above. In a most general embodiment, however, the DNA chip may also be derivated with a single probe oligonucleotide. Preferred probe oligonucleotides are nucleic acid oligomers (DNA, RNA, or PNA fragments) of base length 3 to 50, preferably of length 5 to 30, particularly preferably of length of 7 to 25. According to the present invention, a redox-active substance is bound to the probe oligonucleotides either before or after the latter is bound to the conductive surface.

If the modification of the probe oligonucleotides occurs before the bond to the conductive surface, then the already modifed probe oligonucleotides are bound to the conductive surface as described above. Alternatively, the non-modified probe oligonucleotides bound to the conductive surface are modified with a redox-active substance. at the second, free end of the oligonucleotide chain, directly or indirectly via a spacer.

In both cases, a surface hybrid of the general structure elec-spacer-ss-oligo-spacer-redox (Figure 3) results. The electrical communication between the (conductive) support surface and the redox-active substance ("redox") bridged via a single-strand oligonucleotide in the general structure elec-spacer-ss-oligo-spacer-redox is weak or not present at all. The bridges may, of course, also be carried out without spacers or with only one spacer (elec-ss-oligo-spacer-redox or elec-spacer-ss-oligo-redox).

In a next step, the test sites are brought into contact with the oligonucleotide solution (target) to be examined. Hybridization will only occur if the solution contains

oligonucleotide strands that are complementary to the probe oligonucleotides bound to the conductive surface, or at least widely complementary. In the case of hybridization between the probe and target oligonucleotide, there will be increased conductivity between the support surface and the redox-active substance because these are now bridged via the oligonucleotide composed of a double strand (shown schematically in Figure 3 using an example of the elec-spacer-ss-oligo-spacer-redox).

Because of the change in the electrical communication between the (conductive) support surface and the redox-active substance due to the hybridization of the probe oligonucleotide and the oligonucleotide strand (target) complementary to it, a sequence-specific hybridization event can thus be detected using electrochemical methods such as e.g. cyclic voltammetry, amperometry, or conductivity measurements.

In a particularly preferred embodiment of the present invention, a redox-active substance is used that exhibits regions having a predominantly planar p-π-orbital system extended in a plane, such as e.g. the PQQ of Example 1 (cf. Figure 3), or the quinones of Formula 5 or 7-12 or the porphinoid structures of Formulas 1-4, the pyridine nucleotides of the general Formula 6 and derivatives of these redox-active substances. In this case, the spacer between the nucleic acid oligomer and the redox-active substance is selected in such a way that the plane of the π-orbitals of the redox-active substance can arrange itself parallel to the p-π-orbitals of the base pair of the nucleic acid oligomer hybridized with the complimentary strand and bordering on the redox-active substance. This spatial arrangement of the redox-active substance with partially planar p-π-orbitals extended in a plane proves to be particularly favorable for the electrical conductivity of the double-strand nucleic acid oligomers.

In cyclic voltammetry, the potential of a stationary working electrode is changed linearly as a function of time. Starting at a potential at which no electrooxidation or reduction occurs, the potential is changed until the redox-active substance is oxidized or reduced (i.e. current flows). After running through the oxidation or reduction operation, which produces in the current-voltage curve an initially increasing current, a maximum current (peak), and then a gradually decreasing current, the direction of the potential feed is reversed. The behavior of the products of electrooxidation or electroreduction is then recorded in reverse run.

An alternative electrical detection method, amperometry, is made possible by the fact that the redox-active substance is electrooxidized (electroreduced) by applying a suitable, constant electrode potential, but rereducing (reoxidizing) the redox-active substance to its original state is achieved, not by changing the electrode potential as in cyclic voltammetry, but rather by means of a suitable reducing agent (oxidizing agent) added to the target solution, closing the current circuit of the entire system. As long as reducing agent (oxidizing agent) is present, or as long as the consumed reducing agent (oxidizing agent) is rereduced (reoxidized) on the counter electrode, current flows that can be amperometrically detected and that is proportional to the number of hybridization events .

Brief Description of the Drawings

The invention will be explained in greater detail using the following application example and the accompanying drawings.

Fig. 1	Shows a schematic illustration of the Sanger method of oligonucleotide sequencing;
Fig. 2	Shows a schematic illustration of oligonucleotide sequencing by means of hybridization on a chip;
Fig. 3	Shows a schematic illustration of the surface hybrid of the general structure elec-spacer-ss-oligo-spacer-redox with a 12-bp probe oligonucleotide of the exemplary sequence 5'-TAGTCGGAAGCA-3' (left) and Au-S-ss-oligo-PQQ in the hybridized state as an embodiment example of an elec-spacer-ss-oligo-spacer-redox; only a portion of the probe oligonucleotide having a hybridized complementary strand is shown (right), the attachment of the oligonucleotide to the surface occurred via an -S-CH_2CH_2- spacer, and the attachment of the redox-active substance PQQ occurred via the spacer -CH_2-CH=CH-CO-NH-CH_2-CH_2-NH-;
Fig. 4	Shows a cyclic voltammogram of a test site consisting of Au-S-ss-oligo-PQQ (dotted) compared with an identical test site with completely hybridized target (Au-S-ds-oligo-PQQ, solid line);

Fig. 5	Shows a cyclic voltammogram of a test site with completely hybridized target (Au-S-ds-oligo-PQQ) (solid line) compared with a test site with hybridized target that exhibits 2 base-pair mismatches (Au-S-ds-oligo-PQQ with 2 bp mismatches, broken).

Carrying Out the Invention

An exemplary test site with hybridized target (Au-S-ds-oligo-PQQ) of the general structure elec-spacer-ds-oligo-spacer-redox is shown in Figure 3. In the example of Figure 3, the support surface is a gold electrode. The link between the gold electrode and the probe oligonucleotide was formed with the linker $(HO\text{-}(CH_2)_2\text{-}S)_2$, which was esterified with the terminal phosphate group at the 3' end to $P\text{-}O\text{-}(CH_2)_2\text{-}S\text{-}S\text{-}(CH_2)_2\text{-}OH$ and, following homolytic cleavage of the S-S bond at the gold surface, produced one Au-S bond each, with which 2-hydroxy-mercaptoethanol and mercaptoethanol-bridged oligonucleotide was coadsorbed on the surface. The redox-active substance in the example of Figure 3 is tricarboxylic pyrrolo-quinoline quinone (PQQ) and one of the three carboxylic acid functions of the PQQ (in the example, the C-7-CO_2H function) was used to covalently attach the PQQ to the probe oligonucleotide (amidation and dehydration with the terminal amino function of the $\text{-CH=CH-CO-NH-CH}_2\text{-CH}_2\text{-NH}_2$ spacer attached to the C-5 position of the 5' thymine). Both free, unmodified PQQ and PQQ bridged with the support surface via a short spacer of chain length 1-6, such as e.g. $\text{-S-(CH}_2)_2\text{-NH-}$, or via (modified) double-strand oligonucleotide, e.g. in HEPES buffer with 0.7 molar addition of TEATFB (see abbreviations), is selectively reduced and oxidized in the potential range $0.7\ V \geq \varphi \geq 0.0\ V$, measured against normal hydrogen electrode.

The electrical communication between the (conductive) support surface and the redox pair bridged via single-strand oligonucleotide in the general structure elec-spacer-ss-oligo-spacer-redox is weak or not present at all. For the exemplary test site Au-S-ss-oligo-PQQ (with 12-bp probe oligonucleotides), this is shown with cyclic voltammetry (Figure 4). Without wanting to be bound to a theoretical description, it is assumed that the negative charges of the phosphate skeleton cause a mutual repulsion of the oligonucleotide single strands and thus force a formation of the -spacer-ds-oligo-spacer-redox chain (in the direction of the helix axis) at an angle $\phi < 70°$ to the normal of the support support ("standing tubes"). The (hybridized) test site Au-S-ds-oligo-PQQ of Figure 3 exhibits a formation having $\phi = 30°$. Due to the length of the -spacer-ds-oligo-spacer-redox chain (e.g. approx. 40 Å length of a 12-base-

pair oligonucleotide; the spacers and the attached PQQ are about 10 Å long), if ϕ < 70°, a distance of > 17 Å results between the surface support and the redox-active substance. As a result, the possibility of a direct electron or hole transfer between the support and the redox-active substance can be excluded. By treating the test site(s) with an oligonucleotide solution to be examined, in the case of hybridization between probe and target, there will be increased conductivity between the support surface and the redox pair bridged via a double-strand oligonucleotide. The change in the conductivity manifests itself cyclic voltammetrically in a significant current flow between the support surface and the redox-active substance (Figure 4). It is thus possible to detect the sequence-specific hybridization of the target with the probe oligonucleotides using electrochemical methods such as e.g. cyclic voltammetry.

In addition, defective base pairings (base-pair mismatches) can be recognized by means of a modified cyclic voltammetric characteristic (Figure 5). A mismatch manifests itself in a greater potential difference between the current maximums of electroreduction and electroreoxidation (reversal of electroreduction when potential feed direction is reversed), or electrooxidation and electrorereduction in a cyclic voltammetrically reversible electron transfer process between the electrically conducting support surface and the redox-active substance. This fact has an advantageous effect primarily on amperometric detection because there, the current can be tested at a potential at which the perfectly hybridizing oligonucleotide target supplies significant current, but the defectively paired oligonucleotide target does not. In the example of Figure 5, this is possible at a potential $E\text{-}E_0$ of approx. 0.03 V.

Example 1: Producing the Au-S-ds-oligo-PQQ oligonucleotide electrode. The production of Au-S-ds-oligo-PQQ is divided into 4 subsections, namely producing the support surface, hybridizing the probe oligonucleotide with the complementary double strand (hybridization step), derivatizing the support surface with the double-strand oligonucleotide (incubation step) and attaching the redox-active substance (redox step).

An approx. 100 nm thin gold film on mica (muscovite platelets) forms the support for the covalent attachment of the double-strand oligonucleotides. To this end, freshly cleaved mica was purified with an argon-ion plasma in an electrical discharge chamber and gold (99.99%) was applied, by means of electrical discharge, in a layer thickness of approx. 100 nm. Thereafter, the gold film was freed of surface impurities (oxidation of organic accumulations) with 30% H_2O_2, / 70% H_2SO_4 and immersed in ethanol for approx. 20 minutes to dispel any oxygen adsorbed to the surface. After

rinsing the support surface with bidistilled water, a previously prepared $1x10^{-4}$ molar solution of the (modified) double-strand oligonucleotide is applied to the horizontally disposed surface, such that the entire support surface is moistened (incubation step, see also below).

To prepare the ds oligonucleotide solution, a double-modified 12-bp single-strand oligonucleotide of the sequence 5'-TAGTCGGAAGCA-3' was used, which is esterified with $(HO-(CH_2)_2-S)_2$ at the phosphate group of the 3' end to $P-O-(CH_2)_2-S-S-(CH_2)_2-OH$. At the 5' end, the terminal base of the oligonucleotide, thymine, is modified at the C-5 carbon with $-CH=CH-CO-NH-CH_2-CH_2-NH_2$. A $2x10^{-4}$ molar solution of this oligonucleotide in the hybridization buffer (10 mM Tris, 1 mM EDTA, pH 7.5 with 0.7 molar addition of TEATFB, see abbreviations) was hybridized with a $2x10^{-4}$ molar solution of the (unmodified) complementary strand in the hybridization buffer at room temperature for approx. 2 hours (hybridization step). During a reaction time of approx. 12-24 h, the disulfide spacer $P-O-(CH_2)_2-S-S-(CH_2)_2-OH$ of the oligonucleotide was homolytically cleaved. In this process, the spacer forms a covalent Au-S bond with the Au atoms of the surface, thus causing to a 1:1 coadsorption of the ds-oligonucleotide and the 2-hydroxy-mercaptoethanol.

The gold electrode modified in this way with a dense (1:1) monolayer consisting of ds-oligonucleotide and 2-hydroxy-mercaptoethanol was washed with bidistilled water and subsequently moistened with a solution of $3x10^{-3}$ molar quinone PQQ, 10^{-2} molar EDC, and 10^{-2} molar sulfo-NHS in HEPES buffer. After a reaction time of approx. 1 h, the $-CH=CH-CO-NH-CH_2-CH_2-NH_2$ spacer covalently attaches the PQQ (amidation between the amino group of the spacer and an acid function of the PQQ, redox step).

Resolution of the surface composition with XPS (X-Ray Photoelectron Spectroscopy) showed a maximally densely packed monolayer of 1:1 coadsorbed ds-oligonucleotide and 2-hydroxy-mercaptoethanol (4.7×10^{12} ds-oligonucleotide/cm^2), the long axis (direction of the helix axis) of the ds-oligonucleotides forming an angle of $\phi \approx 30°$ with the surface normal of the gold surface.

Example 2: Producing the Au-S-ss-oligo-PQQ oligonucleotide electrode. Analogously to the production of the Au-S-ds-oligo-PQQ system, the support surface is derivatized with modified single-strand oligonucleotide, dispensing with only the hybridization of the modified oligonucleotide of the sequence 5'-TAGTCGGAAGCA-3' with its complementary strand and, in the incubation step, using only the double-

modified 12-bp single-strand probe oligonucleotide (see Example 1) in the form of a 1 x 10^{-4} molar solution in water and in the presence of 10^{-2} molar Tris, 10^{-3} molar EDTA and 0.7 molar TEATFB (or 1 molar NaCl) at pH 7.5. The redox step was carried out as indicated in Example 1.

Example 3: Producing the Au-S-ds-oligo-PQQ oligonucleotide electrode having 2 bp mismatches. The production of a support surface derivatized with modified double-strand oligonucleotide was carried out analogously to the production of the Au-S-ds-oligo-PQQ system, but only in hybridizing the modified oligonucleotide of the sequence 5'-TAGTCGGAAGCA-3' was a complementary strand used (sequence: 5'-ATCAG***AT***TTCGT-3'), in which bases no. 6 and 7 (counted from the 5' end), which are actually complementary, were modified from C to ***A*** or from C to ***T*** to introduce two base-pair mismatches.

Example 4: Producing an Au-S-ss-oligo-PQQ oligonucleotide electrode having greater inter-oligonucleotide distance. In producing the test sites, care must be taken that, in derivatizing the support surface with single-strand probe oligonucleotide, sufficient space remains between the attached single-strands to allow a hybridization with the target oligonucleotide. To this end, three different methods of proceeding present themselves: (a) Producing an Au-S-ds-oligo-PQQ electrode as described in Example 1, with subsequent thermal dehybridization of the double strands at temperatures of T > 40°C. (b) Producing an Au-ss-oligo-PQQ electrode as described in Example 2, but in the incubation step for derivatizing the gold surface with (double-derivatized) single-strand oligonucleotide, 10^{-5} to 10^{-1} molar 2-hydroxy-mercaptoethanol or another thiol or disulfide linker of suitable chain length is added (depending on the desired inter-oligonucleotide distance) and coadsorbed on the gold surface together with the single-strand oligonucleotide. (c) Producing an Au-ss-oligo-PQQ electrode as described in Example 2, but omitting the 0.7 molar addition of electrolytes (TEATFB in the Example) in the incubation step for derivatizing the gold surface with (double-derivatized) single-strand oligonucleotide. Due to the absence of the salt, the phosphate groups and nitrogen-base atoms of the oligonucleotide are not electrostatically shielded and interact strongly with the gold surface. Because of this, a shallow accumulation of the oligonucleotides results on the electrode surface (ϕ > 60°) and significantly fewer oligonucleotides are bound per surface unit. Thereafter, the oligonucleotides may be returned to the desired position by covalently attaching in a second incubation step (before or after attaching the PQQ) a 2-hydroxy-mercaptoethanol or another thiol or disulfide linker of suitable chain length to the surface gold atoms that are still free. To do this, the electrode that

is less densely covered with single-strand oligonucleotide is moistened, before or after modification with PQQ (Au-S-ss-oligo or Au-S-ss-oligo-PQQ), with an approx. $5x10^{-2}$ molar solution of 2-hydroxy-mercaptoethanol or another thiol or disulfide linker of suitable chain length in ethanol or HEPES buffer (or a mixture thereof, depending on the solubility of the thiol), and incubated for 2-24 h.

Example 5: Carrying out the cyclic voltammetry measurements. The cyclic voltammetry measurements were made using a computer-controlled bipotentiostat (CH Instruments, Model 832) at room temperature in a standard cell having a 3-electrode configuration. The modified gold electrode was used as the working electrode, a platinum wire served as the auxiliary electrode (counter electrode), and an Ag/AgCl electrode with internal saturated KCl solution, separated from the probe space via a Luggin capillary, was used as the reference electrode to determine the potential. Serving as an electrolyte was 0.7 molar TEATFB or 1 molar NaCl. A cyclic voltammogram of the Au-S-ds-oligo-PQQ electrode is shown in Figure 4 in comparison with an Au-S-ss-oligo-PQQ electrode, and the effect of the 2 bp mismatches on the cyclic voltammogram of the Au-S-ds-oligo-PQQ electrode is shown in Figure 5. The potentials are each indicated as $E-E_0$, i.e. relative to the half-wave potential.

In Figure 4, shows clearly a significantly greater current flow as compared with the non-hybridized form is clearly evident when a double-strand oligonucleotide is present. This allows sequence-specific hybridization events to be detected. From Figure 5 it becomes clear that, in the case of hybridization with a target oligonucleotide strand that exhibits 2 base-pair mismatches, for one thing, a weaker current flows, and for another, the difference of the current maximums is increased.

Claims

1. A nucleic acid oligomer modified by attaching a redox-active substance, characterized in that the redox-active substance is selectively oxidizable and reducible at a potential φ, where φ satisfies the condition $2.0\ V \geq \varphi \geq -2.0\ V$, measured against normal hydrogen electrode.

2. The modified nucleic acid oligomer according to claim 1, wherein the redox-active substance is a dye, especially a flavine derivative, a porphyrin derivative, a chlorophyll derivative, or a bacteriochlorophyll derivative.

3. The modified nucleic acid oligomer according to claim 1, wherein the redox-active substance is a quinone, especially a pyrrolo-quinoline quinone (PQQ), a 1,4-benzoquinone, a 1,2-naphthoquinone, a 1,4-naphthoquinone, or a 9,10-anthraquinone.

4. The modified nucleic acid oligomer according to one of the preceding claims, wherein, alternatively, the redox-active substance is covalently attached to one of the phosphoric-acid, carboxylic-acid, or amine moieties, to one of the sugar moieties, or to one of the bases of the nucleic acid oligomer, especially to a terminal moiety of the nucleic acid oligomer.

5. The modified nucleic acid oligomer according to one of claims 1 to 3, wherein the redox-active substance is covalently attached to a branched or linear molecular moiety of any composition and chain length and the branched or linear molecular moiety, alternatively, is attached to one of the phosphoric-acid, carboxylic-acid, or amine moieties, to one of the sugar moieties, or to one of the bases of the nucleic acid oligomer, especially to a terminal moiety of the nucleic acid oligomer.

6. The modified nucleic acid oligomer according to claim 5, wherein the redox-active substance is covalently attached to a branched or linear molecular moiety whose shortest continuous link between the joined structures comprises 1-14 atoms.

7. The modified nucleic acid oligomer according to one of the preceding claims, wherein the modified nucleic acid oligomer can sequence-specifically bind single-strand DNA, RNA, and/or PNA.

8. The modified nucleic acid oligomer according to claim 7, wherein the modified nucleic acid oligomer is a deoxyribonucleic acid oligomer, a ribonucleic acid oligomer, a peptide nucleic acid oligomer, or a nucleic acid oligomer having a structurally analogous backbone.

9. A method of producing a modified nucleic acid oligomer according to claims 1 to 4, characterized in that the redox-active substance is bound to a nucleic acid oligomer, the attachment occurring at a phosphoric-acid or carboxylic-acid group of the nucleic acid oligomer by means of amidation with a (primary or secondary) amino group of the redox-active substance, by means of esterification with a (primary, secondary, or tertiary) alcohol group of the redox-active substance, by means of thioester formation with a (primary, secondary, or tertiary) thioalcohol group of the redox-active substance, or by means of condensation of an amine group of the nucleic acid oligomer with an aldehyde group of the redox-active substance.

10. A method of producing a modified nucleic acid oligomer according to claims 5 to 8, characterized in that the redox-active substance is covalently attached to a branched or linear molecular moiety of any composition and chain length, the attachment occurring at a phosphoric-acid or carboxylic-acid group of the branched or linear molecular moiety by means of amidation with a (primary or secondary) amino group of the redox-active substance, by means of esterification with a (primary, secondary, or tertiary) alcohol group of the redox-active substance, by means of thioester formation with a (primary, secondary, or tertiary) thioalcohol group of the redox-active substance, or by means of condensation of an amine group of the branched or linear molecular moiety with an aldehyde group of the redox-active substance.

11. A modified conductive surface, characterized in that one or more kinds of modified nucleic acid oligomers according to claims 1 to 8 are attached to a conductive surface.

12. The modified conductive surface according to claim 11, wherein the surface consists of a metal or a metal alloy, especially a metal selected from the group

platinum, palladium, gold, cadmium, mercury, nickel, zinc, carbon, silver, copper, iron, lead, aluminum, manganese, and their compounds.

13. The modified conductive surface according to claim 11, wherein the surface consists of a semiconductor, especially a semiconductor selected from the group carbon, silicon, germanium, and α-tin.

14. The modified conductive surface according to claim 11, wherein the surface consists of a binary compound of the elements of groups 14 and 16, a binary compound of the elements of groups 13 and 15, a binary compound of the elements of groups 15 and 16, or a binary compound of the elements of groups 11 and 17, especially a Cu(I)-halide or an Ag(I)-halide.

15. The modified conductive surface according to claim 11, wherein the surface consists of a ternary compound of the elements of groups 11, 13, and 16, or a ternary compound of the elements of groups 12, 13, and 16.

16. The modified conductive surface according to claims 11 to 15, wherein the modified nucleic acid oligomers are attached to the conductive surface covalently or by means of physisorption.

17. The modified conductive surface according to claim 16, wherein, alternatively, one of the phosphoric-acid, carboxylic-acid, or amine moieties, one of the sugar moieties, or one of the bases of the nucleic acid oligomer is attached to the conductive surface, covalently or by means of physisorption, especially to a terminal moiety of the nucleic acid oligomer.

18. The modified conductive surface according to one of claims 11 to 15, wherein branched or linear molecular moieties of any composition and chain length are attached to the conductive surface, covalently or by means of physisorption, and the modified nucleic acid oligomers are covalently attached to these molecular moieties.

19. The modified conductive surface according to claim 18, wherein the branched or linear molecular moiety comprises a shortest continuous link of 1 to 14 atoms between the joined structures.

20. The modified conductive surface according to claims 18 and 19, wherein, alternatively, the branched or linear molecular moiety is covalently bound to one of the phosphoric-acid, carboxylic-acid, or amine moieties, to one of the sugar moieties, or to one of the bases of the nucleic acid oligomer, especially to a terminal moiety of the nucleic acid oligomer.

21. A method of producing a modified conductive surface according to claims 11 to 20, wherein one or more kinds of modified nucleic acid oligomers according to claims 1 to 8 are applied to a conductive surface.

22. The method of producing a modified conductive surface according to claims 11 to 20, wherein one or more kinds of nucleic acid oligomers are bound to a conductive surface and only the nucleic acid oligomers bound to the conductive surface are modified by attaching a redox-active substance to the nucleic acid oligomers.

23. The method of producing a modified conductive surface according to claim 22, wherein the attachment of the redox-active substance to the nucleic acid oligomer occurs by means of reacting the redox-active substance with a phosphoric-acid moiety, a sugar moiety, or one of the bases of the nucleic acid oligomer, especially by means of reaction with a terminal moiety of the nucleic acid oligomer.

24. The method of producing a modified conductive surface according to claim 22, wherein the redox-active substance is covalently attached to a branched or linear molecular moiety of any composition and chain length and the branched or linear molecular moiety, alternatively, is attached to one of the phosphoric-acid, carboxylic-acid, or amine moieties, to one of the sugar moieties, or to one of the bases of the nucleic acid oligomer, especially to a terminal moiety of the nucleic acid oligomer.

25. The method of producing a modified conductive surface according to claims 21 to 24, wherein the nucleic acid oligomer or the modified nucleic acid oligomer is hybridized with the nucleic acid oligomer strand complementary to it and applied to the conductive surface in the form of the double-strand hybrid.

26. The method of producing a modified conductive surface according to claims 21 to 25, wherein the nucleic acid oligomer or the modified nucleic acid oligomer is

applied to the conductive surface in the presence of further chemical compounds that are likewise attached to the conductive surface.

27. A method of electrochemically detecting nucleic acid oligomer hybridization events, characterized in that a conductive surface as defined in claims 11 to 20 is brought into contact with nucleic acid oligomers and, thereafter, detection of the electrical communication between the redox-active moiety and the respective conductive surface occurs.

28. The method according to claim 27, wherein detection occurs by means of cyclic voltammetry, amperometry, or conductivity measurement.

Method of Electrochemically Detecting Nucleic Acid Oligomer Hybrides

Abstract of the Disclosure

The invention relates to a method for the electrochemical detection of sequence-specific nucleic acid oligomer hybridization events. To this end single DNA/RNA/PNA oligomer strands which at one end are covalently joined to a support surface and at the other, free end, covalently linked to a redox pair, are used as hybridization matrix (probe). As a result of treatment with the oligonucleotide solution (target) to be examined, the electric communication between the conductive support surface and the redox pair bridged by the single-strand oligonucleotide, which communication initially is either absent or very weak, is modified. In case of hybridization, the electric communication between the support surface and the redox pair, which is now bridged by a hybridized double-strand oligonucleotide, is increased. This permits the detection of a hybridization event by electrochemical methods such as cyclic voltametry, amperometry or conductivity measurement.

Fig. 1a

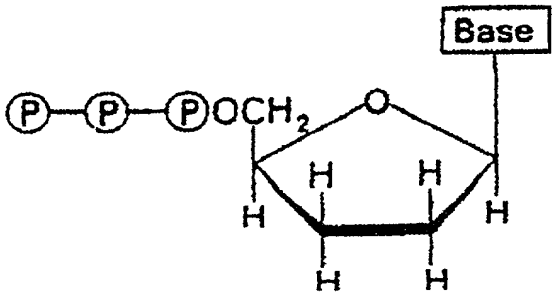


Fig. 1b

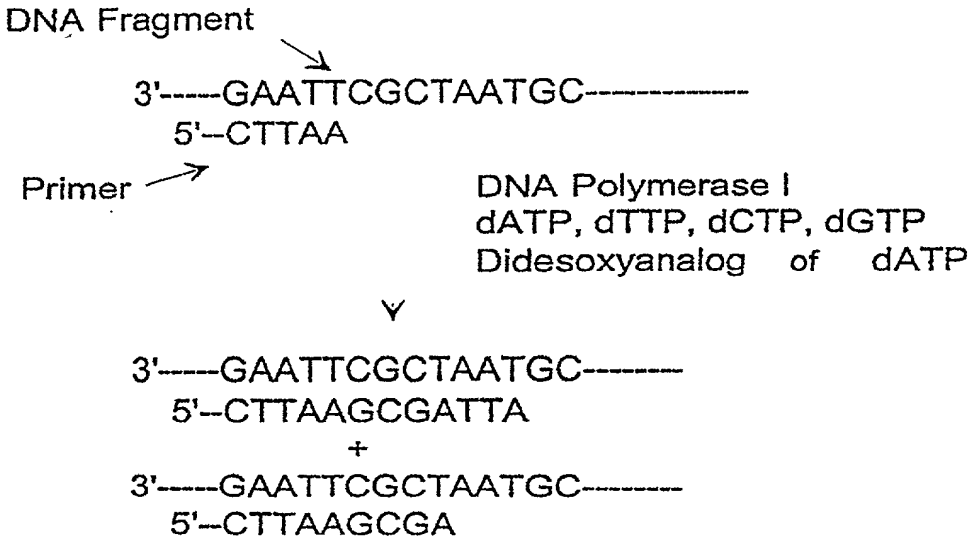


Fig. 1c

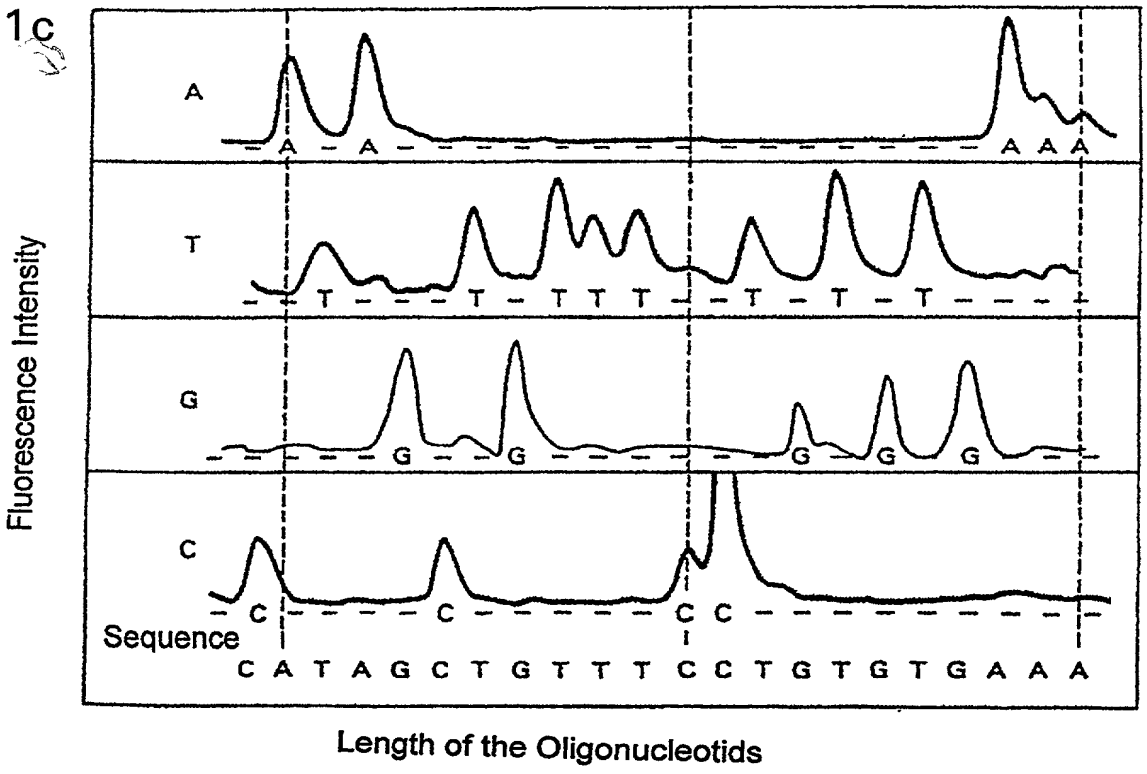
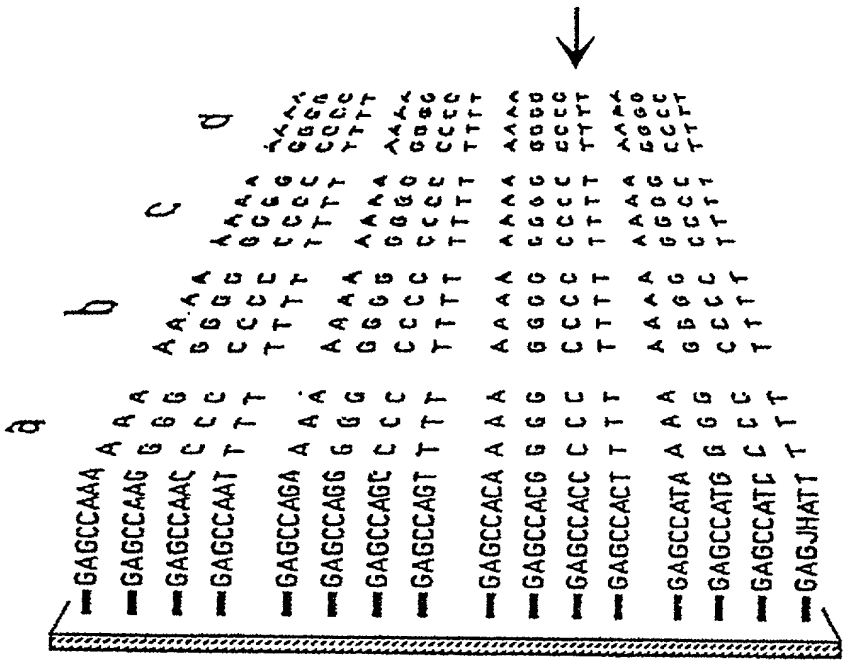


Fig. 2

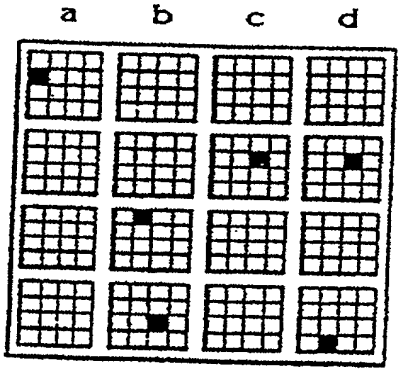
DNA Fragment

5'-...AGTCCCTTGGCTC...-3'



Specific Hybridization on Octamer Matrix

Sequence Determination through Pattern Recognition



3'-TCAGGGAA-5' Octamer 1
3'-CAGGGAAC-5' Octamer 2
3'-AGGGAACC-5' Octamer 3
3'-GGGAACCG-5' Octamer 4
3'-GGAACCGA-5' Octamer 5
3'-GAACCGAG-5' Octamer 6

3'-TCAGGGAACCGAG-5' Composite Complementary Sequence

5'-...AGTCCCTTGGCTC...-3' Deduced Sequence of the DNA Fragment

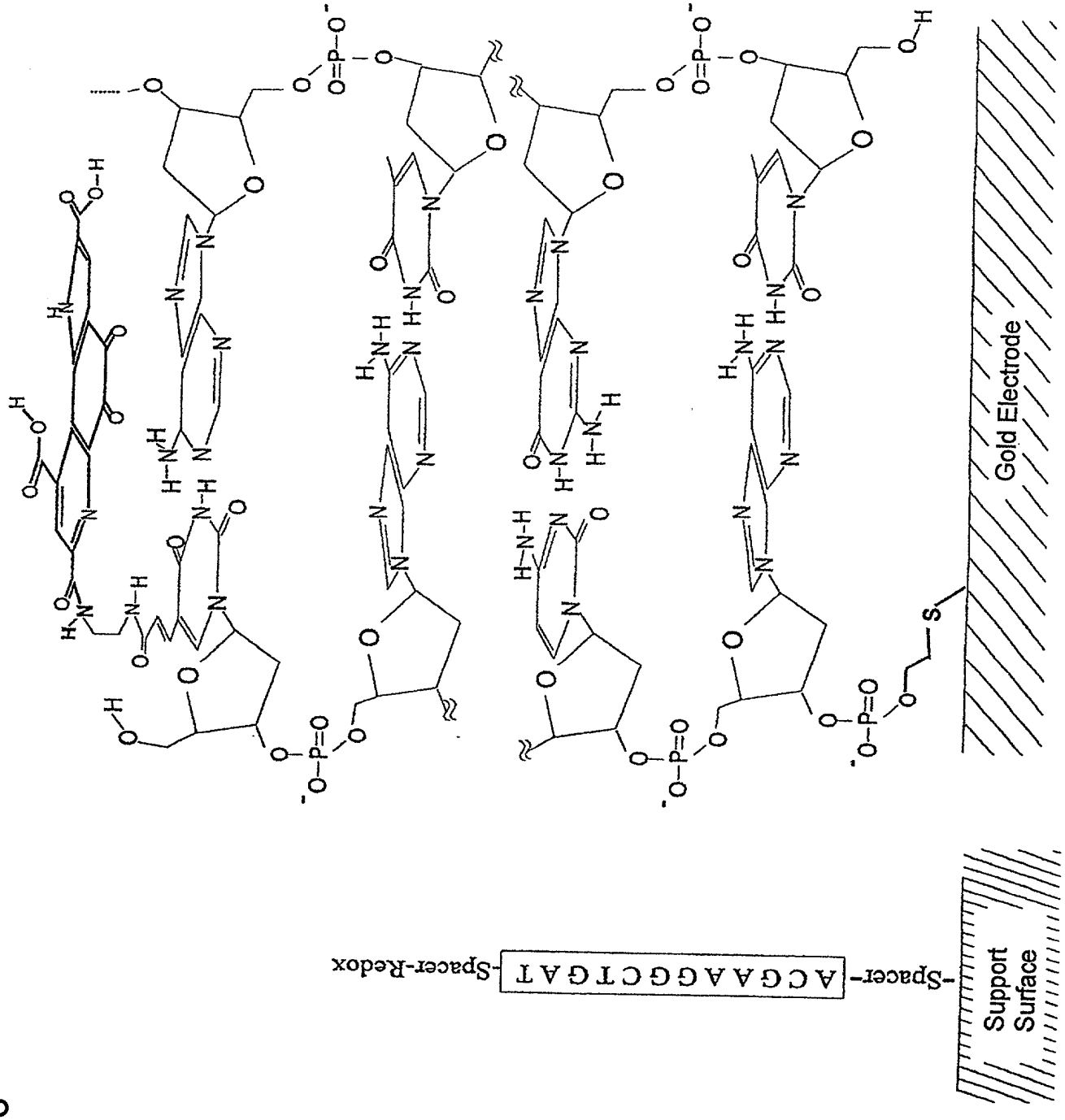
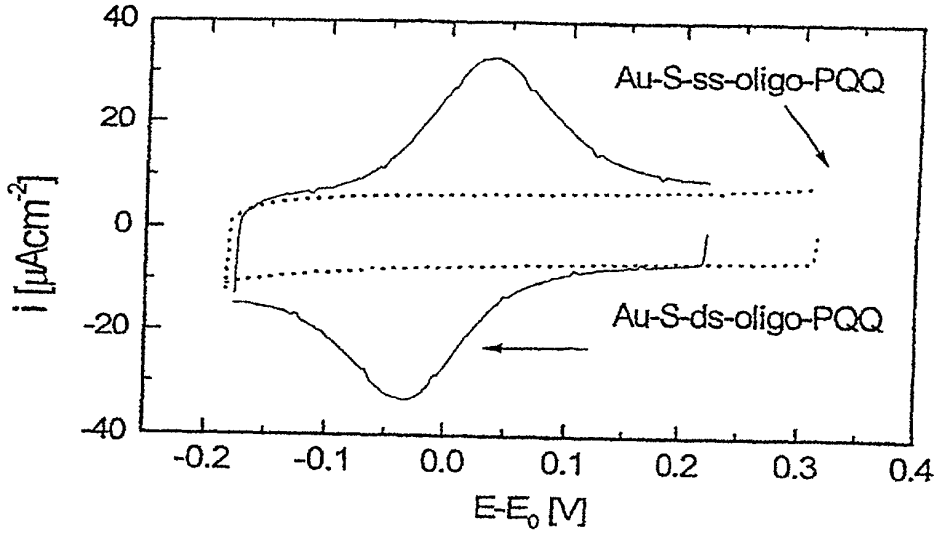

Gold Electrode
Support Surface
-Spacer-
A C G A A G G C T G A T
-Spacer-Redox

Fig. 3

Fig. 4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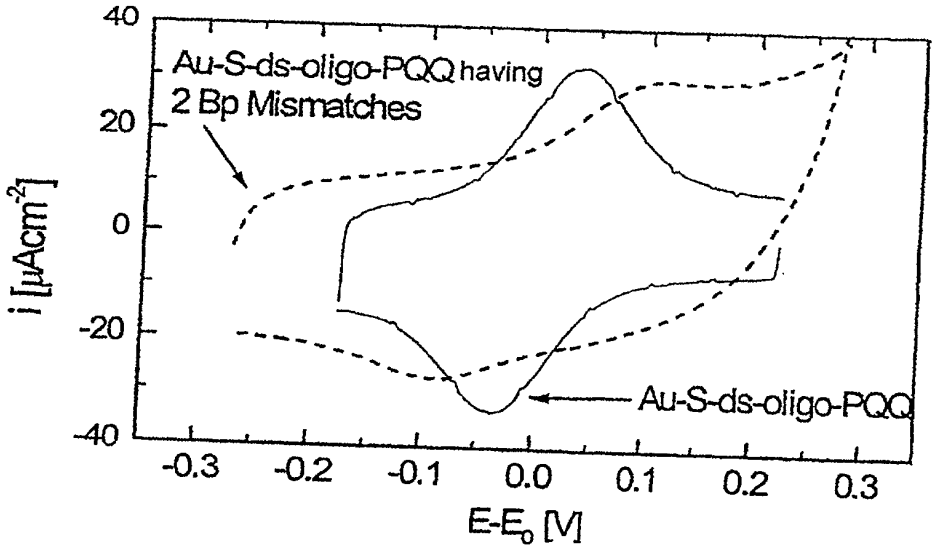
Au-S-ss-oligo-PQQ
Au-S-ds-oligo-PQQ

Fig. 5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E-E0 [V]
Au-S-ds-oligo-PQQ having
2 Bp Mismatches
Au-S-ds-oligo-PQQ

PTO/SB/103 (8-96)
Approved for use through 9/30/98. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Declaration and Power of Attorney for Patent Application
Erklärung für Patentanmeldungen mit Vollmacht

German Language Declaration

Als nachstehend benannter Erfinder erkläre ich hiermit an Eides Statt:

daß mein Wohnsitz, meine Postanschrift und meine Staatsangehörigkeit den im nachstehenden nach meinem Namen aufgeführten Angaben entsprechen, daß ich nach bestem Wissen der ursprüngliche, erste und alleinige Erfinder (falls nachstehend nur ein Name angegeben ist) oder ein ursprünglicher, erster und Miterfinder (falls nachstehend mehrere Namen aufgeführt sind) des Gegenstandes bin, für den dieser Antrag gestellt wird und für den ein Patent für die Erfindung mit folgendem Titel beantragt wird:

Method of Electrochemically detecting Nucleic Acid Oligomer Hybrids

deren Beschreibung hier beigefügt ist, es sei denn (in diesem Falle Zutreffendes bitte ankreuzen), diese Erfindung

☒ wurde angemeldet am 19 Nov. 1999 unter der US-Anmeldenummer oder unter der Internationalen Anmeldenummer im Rahmen des Vertrags über die Zusammenarbeit auf dem Gebiet des Patentwesens (PCT) PCT/EP99/08888 _____ und am _____ abgeändert (falls zutreffend).

Ich bestätige hiermit, daß ich den Inhalt der oben angegebenen Patentanmeldung, einschließlich der Ansprüche, die eventuell durch einen oben erwähnten Zusatzantrag abgeändert wurde, durchgesehen und verstanden habe.

Ich erkenne meine Pflicht zur Offenbarung jeglicher Informationen an, die zur Prüfung der Patentfähigkeit in Einklang mit Titel 37, Code of Federal Regulations, § 1.56 von Belang sind.

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

_____

_____

the specification of which is attached hereto unless the following box is checked:

☐ was filed on _____ as United States Application Number or PCT International Application Number _____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

PTO/SB/103 (8-96)
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Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

German Language Declaration

Ich beanspruche hiermit ausländische Prioritätsvorteile gemäß Title 35, US-Code, § 119 (a)-(d), bzw. § 365(b) aller unten aufgeführten Auslandsanmeldungen für Patente oder Erfinderurkunden, oder § 365(a) aller PCT internationalen Anmeldungen, welche wenigstens ein Land ausser den Vereinigten Staaten von Amerika benennen, und habe nachstehend durch ankreuzen sämtliche Auslands- anmeldungen für Patente bzw. Erfinderurkunden oder PCT internationale Anmeldungen angegeben, deren Anmeldetag dem der Anmeldung, für welche Priorität beansprucht wird, vorangeht.

I hereby claim foreign priority under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Applications
(Frühere ausländische Anmeldungen)

Priority Not Claimed
Priorität nicht beansprucht

(Number) (Nummer)	(Country) (Land)	(Day/Month/Year Filed) (Tag/Monat/Jahr der Anmeldung)	
19853957.6	Germany	23 Nov. 1998	☐
19921940.0	Germany	29 Apr. 1999	☐

Ich beanspruche hiermit Prioritätsvorteile unter Title 35, US-Code, § 119(e) aller US-Hilfsanmeldungen wie unten aufgezählt.

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s)listed below.

(Application No.) (Aktenzeichen)	(Filing Date) (Anmeldetag)

Ich beanspruche hiermit die mir unter Title 35, US-Code, § 120 zustehenden Vorteile aller unten aufgeführten US-Patentanmeldungen bzw. § 365(c) aller PCT internationalen Anmeldungen, welche die Vereinigten Staaten von Amerika benennen, und erkenne, insofern der Gegenstand eines jeden früheren Anspruchs dieser Patentanmeldung nicht in einer US-Patentanmeldung, bzw. PCT internationalen Anmeldung in in einer gemäß dem ersten Absatz von Title 35, US-Code, § 112 vorgeschriebenen Art und Weise offenbart wurde, meine Pflicht zur Offenbarung jeglicher Informationen an, die zur Prüfung der Patentfähigkeit in Einklang mit Title 37, Code of Federal Regulations, § 1.56 von Belang sind und die im Zeitraum zwischen dem Anmeldetag der früheren Patentanmeldung und dem nationalen oder im Rahmen des Vertrags über die Zusammenarbeit auf dem Gebiet des Patentwesen (PCT) gültigen internationalen Anmeldetags bekannt geworden sind.

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(Application No.) (Aktenzeichen)	(Filing Date) (Anmeldetag)	(Status) (patented, pending, abandoned) (Status) (patentiert, schwebend, aufgegeben)
PCT/EP99/08888	11/19/99	

Ich erkläre hiermit, daß alle in der vorliegenden Erklärung von mir gemachten Angaben nach bestem Wissen und Gewissen der Wahrheit entsprechen, und ferner daß ich diese eidesstattliche Erklärung in Kenntnis dessen ablege, daß wissentlich und vorsätzlich falsche Angaben oder dergleichen gemäß § 1001, Title 18 des US-Code strafbar sind und mit Geldstrafe und/oder Gefängnis bestraft werden können und daß derartige wissentlich und vorsätzlich falsche Angaben die Rechtswirksamkeit der vorliegenden Patentanmeldung oder eines aufgrund deren erteilten Patentes gefährden können.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

German Language Declaration

VERTRETUNGSVOLLMACHT: Als benannter Erfinder beauftrage ich hiermit den (die) nachstehend aufgeführten Patentanwalt (Patentanwälte) und/oder Vertreter mit der Verfolgung der vorliegenden Patentanmeldung sowie mit der Abwicklung aller damit verbundenen Angelegenheiten vor dem US-Patent- und Markenamt: *(Name(n) und Registrationsnummer(n) auflisten)*

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: *(list name and registration number)*

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Vor- und Zuname des einzigen oder ersten Erfinders Gerhard HARTWICH	Full name of sole or first inventor
Unterschrift des Erfinders Datum 04/05/01	Inventor's signature Date
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Staatsangehörigkeit Deutsch	Citizenship
Postanschrift Nibelungenstr. 10	Post Office Address
D-80638 München Germany DEX	
Vor- und Zuname des zweiten Miterfinders (falls zutreffend)	Full name of second joint inventor, if any Adam HELLER
Unterschrift des zweiten Erfinders Datum	Second Inventor's signature Date
Wohnsitz	Residence 7531 Velburn Circle, Austin, Texas 78731, U.S.A.
Staatsangehörigkeit	Citizenship U.S.A.
Postanschrift	Post Office Address SAME AS ABOVE

(Im Falle dritter und weiterer Miterfinder sind die entsprechenden Informationen und Unterschriften hinzuzufügen.)

(Supply similar information and signature for third and subsequent joint inventors.)

PATKRI P01AUS FRI042/01

COMBINED DECLARATION AND POWER OF ATTORNEY

(Original, Design, National Stage of PCT, Supplemental)

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is of the following type: (check one applicable item below)

- ☐ original
- ☐ design
- ☐ supplemental
- ■ National Stage of PCT
- ☐ divisional (see added page)
- ☐ continuation (see added page)
- ☐ continuation-in-part (see added page)

INVENTORSHIP IDENTIFICATION

My/our residence, post office address and citizenship is/are as stated below next to my/our name. I/We believe that the named inventor or inventors listed below is/are the original and first inventor or inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

METHOD FOR THE ELECTROCHEMICAL DETECTION OF NUCLEIC ACID OLIGOMER HYBRIDS

SPECIFICATION IDENTIFICATION

The specification of which: (complete (a), (b) or (c))

(a) ☐ is attached hereto.

(b) ☐ was filed on _____ as
☐ Serial No. _____ or
☐ Express Mail No. _____ as Serial No. (not yet known) and was amended on _____(if applicable).

(c) ■ was described and claimed in PCT International Application No. PCT/EP99/08888 filed on November 19, 1999 and as amended under PCT Article 19 on _____ (if any).

(d) ☐ amended on _____

POWER OF ATTORNEY

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name(s) and registration number(s))

2

Anthony G. M. Davis — Registration No. 27,868
Michael J. Bujold — Registration No. 32,018
Scott A. Daniels — Registration No. 42,462

☐ Attached as part of this Declaration and Power of Attorney is the authorization of the above-named attorney(s) to accept and follow instructions from my representative(s).

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ACKNOWLEDGMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

We hereby state that I/we have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose to the United States Patent Office all information which is known to be material to patentability of this application as defined in § 1.56 of Title 37 of the Code of Federal Regulations.

PRIORITY CLAIM

We hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me/us on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

COUNTRY	APPLICATION NO.	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 37 USC 119
Germany	198 53 957.6	November 23, 1998	■YES □NO
Germany	199 21 940.0	April 29, 1999	■YES □NO

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

□ We hereby claim the benefit, under 35 U.S.C. 119(e), of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YY)	□ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

DECLARATION

We hereby declare that all statements made herein of my/our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of first joint inventor: Gerhard HARTWICH

Inventor's signature: _____ Date: _____

Residence: Nibelungenstraße 10, D-80639 München, FED REP GERMANY

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2nd

Full name of second joint inventor: Adam HELLER

Inventor's signature: Adam Heller Date: Nov 26, 01

Residence: 7531 Velburn Circle, Austin, Texas 78731, United States of America

Post Office Address: Same as above TX Country of Citizenship: U.S.A.

IMPORTANT NOTICE RE
DUTY OF CANDOR AND GOOD FAITH

The Duty of Disclosure requirements of Section 1.56(a), of Title 27 of the Code of Federal Regulations, are as follows:

A duty of candor and good faith toward the Patent and Trademark Office rests on the inventor, on each attorney or agent who prepares or prosecutes the application, and on every other individual who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application. All such individuals have a duty to disclose to the Patent Office all information they are aware of which is known to be material to patentability of the application. Such information is material where there is a substantial likelihood that a reasonable examiner would consider it important in deciding whether to allow the application to issue as a patent. The duty is commensurate with the degree of involvement in the preparation or prosecution of the application.

By virtue of this regulation, each inventor executing the Declaration for the filing of a patent application acknowledges his/her duty to disclose information of which he/she is aware and which may be material to the examination of the application.

Inherent in this is the duty to disclose any knowledge or belief that the invention:

(a) was ever known or used in the United States of America before his/her invention thereof;

(b) was patented or described in any printed publication in any country before his/her invention thereof or more than one year prior to the actual filing date of the United States patent application;

(c) was in public use or on sale in the United States more than one (1) year prior to the actual filing date of the United States patent application; or

(d) has been patented or made the subject of inventor's certificate issued before the actual filing date of the United States patent application in any country foreign to the United States on an application filed by him/her or his/her legal representative(s) or assign(s) more than twelve (12) months before the actual filing date in the United States.

NOTE: The "Information" concerned includes, but is not limited to, all published applications and patents, including applicant(s) and assignee(s) own, United States or foreign application(s) and patent(s), as well as any other pertinent prior art known, or which becomes known, to the inventor or his/her representative(s). Where English language equivalents of foreign language documents are known, they should be identified and, when possible, copies supplied. Failure to comply with this requirement may result in a patent issued on the application being held invalid even if the known prior art which is not supplied is material to only one claim of that patent.

If there is any doubt concerning whether or not a citation is "material" to patentability of the application, it is better to err on the side of safety and disclose such art to the United States Patent Office.